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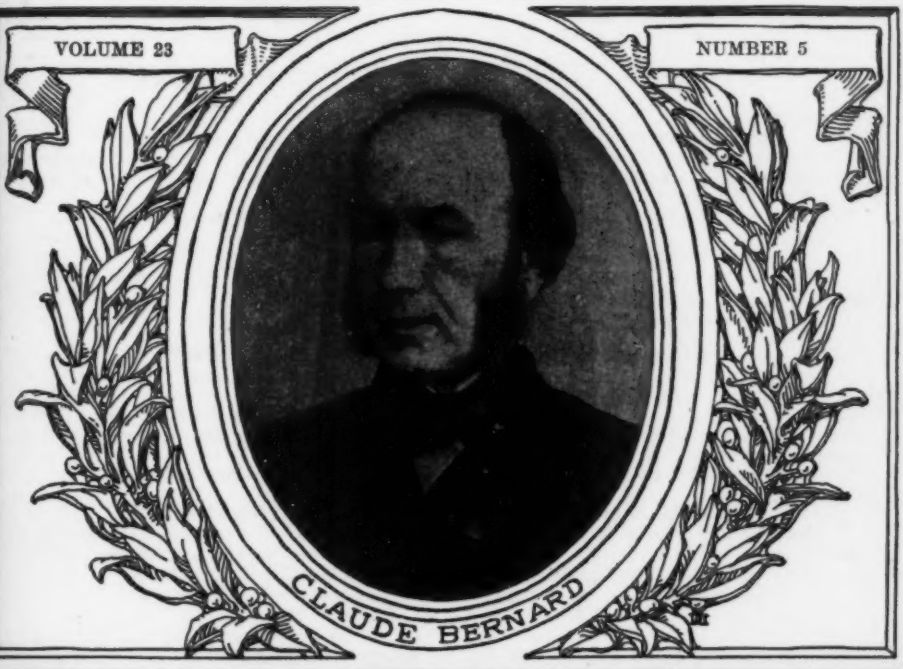

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THE EFFECT OF AROMATIC HYDROCARBONS ON THE GROWTH OF YOUNG RATS

HAROLD D. WEST AND NELSON C. JEFFERSON
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ONE CHART

(Received for publication January 19, 1942)

In a preliminary communication (West, '40) it was reported that complete cessation of growth is caused by the introduction of 1% diphenyl into a 6% casein diet fed to young rats. The condition is relieved and growth is resumed when either l-cystine or dl-methionine is added to the diet. It was supposed that the hydrocarbon is detoxicated through a mechanism involving the sulfur-containing amino acids.

In view of the fact that the decreased food consumption observed in the experimental animals might conceivably be interpreted as causing the lowered growth rate, and further, since the improvement in food consumption and growth following addition of the sulfur-containing amino acids to the hydrocarbon diet might be attributed to the effect of these acids in increasing the nutritive value of a casein diet, it was thought that experiments designed to rule out these factors as being responsible for the results previously observed should be undertaken. These as well as similar experiments with chrysene are here presented.

EXPERIMENTAL

The basal diet contained in per cent: casein, 6; salt mixture (Osborne and Mendel, '19), 4; sucrose, 15; cod liver oil, 1; hydrogenated cottonseed oil [1], 24; and corn starch, 50. The

[1] Crisco.

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quantity of starch added to the supplemented diets was reduced by amounts equivalent to the weight of the material added. Experiments were carried out with supplements of either (a) hydrocarbons alone (0.3% diphenyl; 0.3% chrysene), (b) amino acids alone (0.120% l-cystine; 0.150% dl-methionine), or (c) both hydrocarbons and amino acids [2].

Young white rats from our stock colony were placed on each of the diets, respectively, shortly after weaning. They were kept in raised-bottom cages and allowed water and food ad libitum. The experiments lasted 30 days. Each rat received daily 0.4 gm. of brewers' yeast [3].

In a second series of experiments paired-feeding tests were carried out with litter mates on different diets.

RESULTS

The results of the ad libitum experiments are shown in chart 1. Both diphenyl and chrysene retarded the growth rate in the amounts given, chrysene being somewhat more active in this respect. Addition of either l-cystine or dl-methionine, to the basal diet increased both the rate of growth and the food consumption. Diets containing both hydrocarbon and amino acid gave intermediate values.

The paired-feeding experiments (table 1) eliminated the differences in food consumption but showed the same definite trend observed in growth rates in the ad libitum experiments.

DISCUSSION

The results of the experiments demonstrate that depression of growth by the hydrocarbons is not due to the decreased food consumption. The hydrocarbons evidently react in some manner with the sulfur-containing amino acids since growth rates

[2] The l-cystine was C. P. Cystine purchased from the Coleman and Bell Company. It was recrystallized and analyzed. The dl-methionine was purchased from the Research Laboratory, Eastman Kodak Company. The diphenyl was a pure sample melting at 69° C. The chrysene was kindly furnished us by Dr. N. O. Calloway. It had been recrystallized ten times and melted at 247° C. (Melting points are uncorrected.)

[3] Mead's.

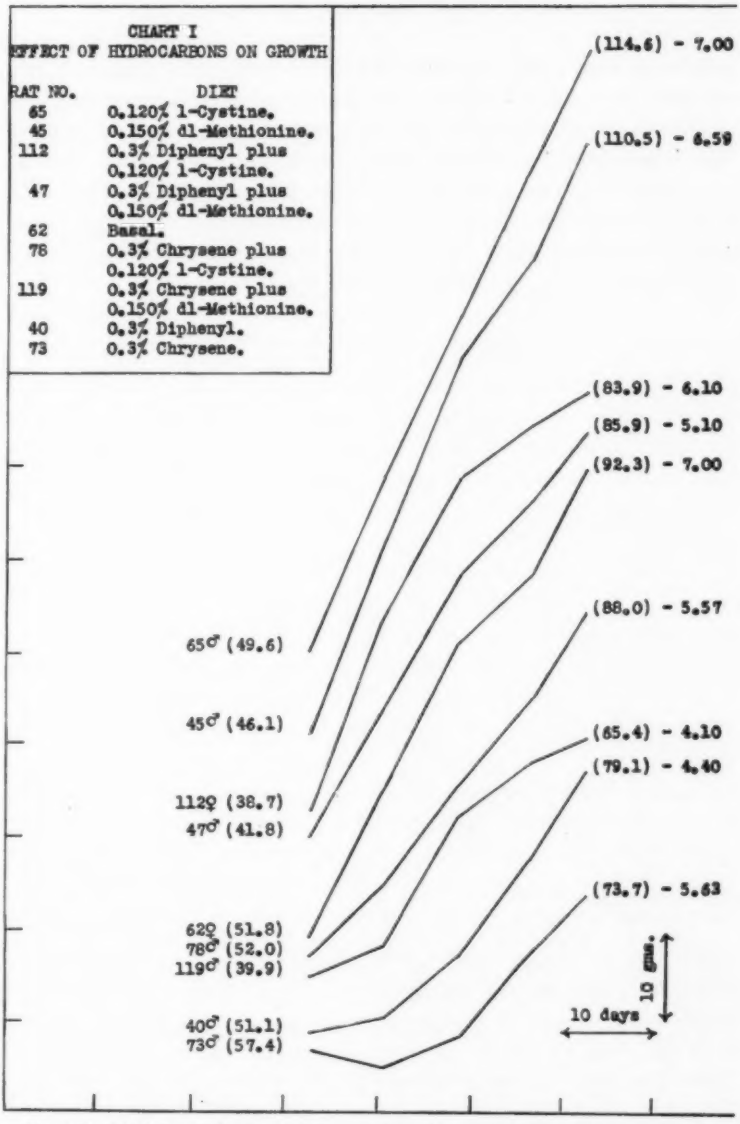

Chart 1 Initial and final weights are given in parentheses. The average daily food consumption in grams is placed after the final weight. Each curve is typical of results with at least three to five animals.

intermediate between those observed for the hydrocarbon supplements alone and for the amino acid supplements alone were obtained when the aromatic compounds were added to the same diet. Another argument which points toward the fundamental nature of the phenomenon is based on the fact that varying the amount of hydrocarbon gave results which varied directly with the quantity fed. When the basal diet

TABLE 1

Growth of young rats for 30 days on the basal diet with and without supplements. Paired-feeding experiments.

DIET	RAT NO.	SEX	BODY WEIGHT			DAILY FOOD INTAKE
			Initial	Final	Daily change	
Basal	61	♀	55.6	90.3	1.15	3.80
0.3% Diphenyl	60	♀	50.3	76.1	0.83	3.80
Basal	86	♀	59.3	107.5	1.60	6.37
0.3% Diphenyl	85	♀	71.6	99.6	0.93	6.37
Basal	98	♂	41.3	80.6	1.26	6.51
0.120% l-Cystine	99	♂	46.4	100.0	1.73	6.51
Basal	100	♀	40.3	81.2	1.31	6.43
0.120% l-Cystine	101	♀	40.7	94.7	1.74	6.43
Basal	102	♂	67.9	102.0	1.16	7.03
0.150% dl-Methionine	103	♂	69.9	121.0	1.73	7.03
Basal	104	♂	66.7	103.7	1.23	6.93
0.150% dl-Methionine	105	♀	61.2	117.1	1.86	6.93
Basal	107	♀	64.2	89.2	0.86	5.49
0.3% Chrysene	106	♀	61.0	76.4	0.51	5.49
Basal	109	♀	60.2	86.2	0.86	5.08
0.3% Chrysene	108	♀	63.7	75.1	0.38	5.08

was supplemented with 0.2% diphenyl the average daily change in weight for two animals in 30 days was 0.99 gm.; with 0.3%, 0.63 gm.; with 0.4%, 0.55 gm.; and with 0.5%, 0.39 gm. Supplements of 0.2% and 0.5% chrysene allowed a daily average increase in weight of 0.79 gm. and 0.52 gm., respectively.

The experimental results indicate that chrysene is somewhat more active than diphenyl in depressing the growth of

the animals. The reason for this difference is not apparent at the present time. Chrysene may be more toxic than diphenyl. Also, since chrysene is known to be very mildly carcinogenic (Cook, Haslewood, Hewett, Hieger, Kennaway and Mayneord, '27) this may be a factor (Haddow, Scott and Scott, '37), but sufficient proof of the contention that carcinogenic compounds are more active than non-carcinogenic compounds in retarding the growth of rats on a low protein diet is still lacking (White and White, '39).

Attempts in this laboratory to demonstrate the presence of a sulfur-containing detoxication product in the urine of rats fed diphenyl and chrysene have so far met with failure. However, some evidence has been adduced for the fact that one detoxication product of diphenyl is a crystalline phenol. These results will be the subject of a subsequent report. Of interest in this connection is the finding of Stroud ('40) that a yield of 25.4% of 4-hydroxydiphenyl can be achieved in isolation experiments using the urine of rabbits fed diphenyl.

While the growth experiments clearly indicate that the sulfur-containing amino acids are involved in the metabolism of the hydrocarbons it does not seem mandatory that the final detoxication product should contain sulfur; nor does the proof of the mediation of these amino acids in the detoxication process call for the isolation of a mercapturic acid or similar derivatives since it has been shown that synthetic sulfhydryl and cysteine derivatives of certain carcinogenic compounds are quite unstable, being easily convertible even in neutral aqueous medium to the corresponding disulfides (Wood and Fieser, '40). The products could then conceivably yield the corresponding carboxyl or hydroxyl derivatives (Stekol, '40).

CONCLUSIONS

1. Both diphenyl and chrysene retard the growth of young rats on a low protein diet.

2. This condition is relieved by introduction of either l-cystine or dl-methionine into the hydrocarbon diets.

3. By employing the method of paired-feeding it has been shown that the depression of growth of the animals on the hydrocarbon diets is not due to the lowered food consumption observed in the ad libitum experiments.

4. The paired-feeding experiments also strengthen the conclusion that the alleviation of the condition imposed by the hydrocarbons is not due to the effect of the sulfur-containing amino acids in increasing the nutritive value of the basal diet but to the increased demands of the tissues for these acids under the experimental conditions.

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THE FATE OF EXCESS VITAMIN A STORES DURING DEPLETION

VALUE OF THE HISTOLOGIC DEMONSTRATION OF VITAMIN A [1]

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ONE PLATE (EIGHT FIGURES)

(Received for publication December 6, 1941)

Studies on the histologic demonstration of vitamin A in rat organs by means of fluorescence microscopy (Querner, '35; Popper, '40) have shown that high values for vitamin A fluorescence are seen in the livers of animals that have received large amounts of the vitamin (Popper and Greenberg, '41). The fluorescence is chiefly visible in the endothelial lining of the blood sinusoids of the liver, in the Kupffer cells, and to only a slight extent in the epithelial cells. Davies and Moore ('35) reported that when excessive amounts of vitamin A were fed, a rat liver could store enough of the vitamin to maintain the animal for a century. If such a rat was kept on a vitamin A deficient diet this enormous amount of vitamin A was quickly lost. In a later publication, Moore ('40) related this rapid drop in the initial depletion time of such rats to a lack of vitamin E. This non-economic utilization of vitamin A when given in huge amounts could be explained by the histologic picture: the Kupffer cells store the excess of vitamin A and the liver cells constitute the only permanent storage place in the body. The excess seen in the Kupffer

[1] This work was supported by a grant from the Committee on Scientific Research of the American Medical Association.

cells may either be destroyed there or given up to other organs by way of the blood stream.

To study these possibilities we have repeated the experiments of Davies and Moore. In addition to the chemical assay of blood and livers for vitamin A the changes in the histologic distribution were observed in rats first given large amounts of vitamin A and then placed on a vitamin A deficient diet. It was hoped to throw some light on the role of the Kupffer cells both in hypervitaminosis and during deficiency since the importance of these cells in vitamin A metabolism is indicated (Uotila and Simola, '38; Hirt and Wimmer, '40; Popper and Greenberg, '41).

In this study chemical and histologic examinations of the liver with various gradations of the vitamin A content were made; this gave us a chance to evaluate the histologic method for demonstration of vitamin A.

MATERIAL AND METHODS

Out of ten litters, fifty-three rats were used which were as close to 4 weeks of age as possible with a maximum age variation of 6 days. Their mothers had been fed Sherman's diet 13. These animals were given large amounts of vitamin A prior to depletion by supplementing diet 13 with a vitamin A concentrate administered orally. During the first week each rat received 20,000 I.U. of vitamin A [2] daily. This dosage was discontinued due to the development of the toxic signs of hypervitaminosis A (Drigalski, '33; Domagk and Dobeneck, '33); after 3 days 10,000 to 12,000 I.U. of vitamin A [3] were administered daily for 28 days. Two male and two female rats were then sacrificed. The livers were examined histologically and chemically for the vitamin A content; the eyes were examined histologically and the pooled blood serum chemically for the vitamin A content. The remaining rats were placed on a vitamin A free diet and at suitable intervals groups of four animals (two male and two female) nearly alike

[2] Shark liver oil concentrates of Vitamin Inc., Chicago, containing approximately 1,000,000 I.U. of vitamin A and 3333 I.U. of vitamin D in 1 gm., were used after dilution with cottonseed oil.

[3] Halibut liver oil contributed by Mead Johnson Company.

in weight and age were sacrificed and the analyses made.[4] Some of the eyes were examined after removal under dark red light following 12 hours of dark adaptation; others after light adaptation.

The chemical method used for determination of vitamin A was that of McCoord and Luce-Clausen ('34), using the Evelyn Photoelectric Colorimeter. The values were recorded as Evelyn Photoelectric Colorimeter Units.[5]

Parts of the livers and the eyes were fixed for 12 hours in formaldehyde solution (U.S.P.). Frozen sections were mounted in water and then examined under the fluorescence microscope. The details of the technic are described elsewhere (Popper, '41). Vitamin A imparts a bright green fluorescence which is quickly destroyed by ultra violet light. This fading fluorescence is imparted by lipoids in tissue sections in a degree parallel to the amount of vitamin A present. A mercury vapor bulb, rich in ultra violet rays, serves as the light source. A filter system absorbs the visible light. A standard microscope is used. Fluorescence tissue constituents change the ultra violet light to various colors of visible light. The remaining ultra violet rays are absorbed by a filter over the eye piece. Since many tissue constituents impart some kind of fluorescence, the orientation is almost as easy in an unstained section under ultra violet light as it is in a stained section in visible light. The vitamin A fluorescence is differentiated from other kinds of fluorescence by the green color, the brightness and the fading. In addition, frozen sections stained with methylene blue and with phosphin 3R, a sensitive fat stain, were examined. In the first an exact localization of the fluorescence is possible if the ultra violet filter is changed to a ground glass filter. Photomicrographs were taken with a small film camera (Popper and Elsasser, '41).

[4] When one rat could not provide enough serum, the blood of two in the group was pooled. The last two groups consisted of two and three rats, respectively.

[5] The liver and blood values refer to Evelyn Photoelectric Colorimeter units per 100 gm. liver and per 100 cc. of serum, respectively. We found an average of 235 units per 100 gm. liver and 10.2 units per 100 cc. of serum in adult stock animals on diet 13 (Brenner et al., '42).

RESULTS

The results obtained are shown in table 1 in which the total amount of fluorescence in the liver is indicated by crosses. Even with a depletion period of 30 weeks no complete exhaustion of the vitamin A depots was found with the chemical and histologic method. Through the entire range from "hypervitaminosis" down to almost avitaminosis the chemical assay corresponded with the amount of fluorescence. In the lower ranges the parallelism between histologic and chemical methods was more striking than was indicated by the rough way of marking with crosses. In the highest ranges (above 7000 units) the histologic picture only revealed large amounts of vitamin A without definite differences.

The variations of the histologic picture were characteristic. In the hypervitaminotic stage the Kupffer cells were extremely rich in the fluorescence and irregular in shape (fig. 1). The fading required several minutes and after that a dim bluish white fluorescence of the Kupffer cells remained indicating a high fat content. The liver cells revealed a moderate vitamin A fluorescence localized in fine lipoid droplets which lined the edge of the cells like pearls on a string. Their cytoplasm imparted a diffuse, much dimmer, green fading fluorescence. The Kupffer cell fluorescence exceeded by far that of the liver cells. In the periportal fields and the areas around the central veins fine droplets with vitamin A fluorescence were seen, apparently in endothelial and other connective tissue cells. The vitamin A fluorescence was homogenous throughout the lobules.

Starting from the second week the total amount of fluorescence diminished, due to a decrease in the Kupffer cell fluorescence. These cells appeared less irregular and their fluorescence faded more quickly and completely (fig. 2). When the chemical value dropped below 4000 to 5000 units the prevalence of the fluorescence in the Kupffer cells disappeared. The liver cell fluorescence remained unchanged; one even gained the impression that it increased, although this may have been an optical illusion. Somewhat later the

TABLE 1

Comparison of the vitamin A content of blood and liver (determined chemically) with the vitamin A fluorescence of the liver in hypervitaminotic rats on a vitamin A deficient diet.

TIME OF DEPLETION	SEX	VITAMIN A FLUORESCENCE [1]				CHEMICAL ASSAY FOR VITAMIN A	
		Total amount	Liver cells	Kupffer cells	Localization	Liver	Blood serum
						Units/ 100 gm.	*Units/ 100 cc.*
0	F.	+++++	+++	+++++	Diffuse	12,931	40
0	F.	+++++	+++	+++++	Diffuse	11,748	
0	M.	+++++	+++	+++++	Diffuse	10,768	
0	M.	+++++	+++	+++++	Diffuse	7,498	
3 days	F.	+++++	+++	+++++	Diffuse	14,628	
3 days	M.	+++++	+++	+++++	Diffuse	7,661	16.9
3 days	F.	+++++	+++	+++++	Diffuse	16,674	
3 days	M.	++++	+++	+++++	Diffuse	7,714	20.4
1 week	F.	+++++	+++	+++++	Diffuse	19,659	
1 week	M.	+++++	+++	+++++	Diffuse	14,420	20.2
1 week	F.	+++++	+++	+++++	Diffuse	14,533	
1 week	M.	++++	+++	++++	Diffuse	11,735	17.2
2 weeks	F.	++++	+++	++++	Diffuse	5,733	
2 weeks	F.	++++	+++	++++	Diffuse	6,473	11.5
2 weeks	M.	+++	+++	+++	Diffuse	5,525	14.0
2 weeks	M.	++	+++	++	Diffuse	1,801	12.4
3 weeks	F.	++++	+++	++++	Diffuse	4,544	
3 weeks	F.	++++	+++	++++	Diffuse	4,293	9.4
3 weeks	M.	++++	+++	+++	Diffuse	4,413	
3 weeks	M.	++++	+++	+++	Diffuse	5,762	19.8
4 weeks	F.	++++	+++	+++	Diffuse	4,938	
4 weeks	F.	+++	+++	++	Diffuse	2,562	8.8
4 weeks	M.	++	++	++	Diffuse	1,570	
4 weeks	M.	++	+++	++	Diffuse	1,767	14.7
5 weeks	F.	++++	+++	+++	Diffuse	2,968	
5 weeks	F.	++++	+++	++++	Diffuse	4,597	13.1
5 weeks	M.	++	++	++	Central	759	14.9
5 weeks	M.	+++	+++	++	Diffuse	2,919	19.0
6 weeks	F.	+++	+++	++	Diffuse	1,948	
6 weeks	F.	++++	+++	++++	Diffuse	4,547	7.7
6 weeks	M.	++	++	++	Peripheral	717	10.8
6 weeks	M.	++	++	++	Peripheral	980	16.3
8 weeks	F.	+++	+++	+++	Diffuse	1,816	
8 weeks	F.	+++	+++	+++	Diffuse	1,950	8.8
8 weeks	M.	++	++	+	Diffuse	694	14.9
8 weeks	M.	++	++	+	Diffuse	674	9.8
10 weeks	F.	++	++	+	Diffuse	965	
10 weeks	F.	+++	++	++	Diffuse	1,265	8.6
10 weeks	M.	+	+	++	Peripheral	238	16.1
10 weeks	M.	++	++	++	Peripheral	350	14.7
13 weeks	F.	++	++	+	Diffuse	863	
13 weeks	F.	+++	++	++	Diffuse	2,458	6.5
13 weeks	M.	+	±	+	Central	228	
13 weeks	M.	+	±	+	Central	112	10.5
18 weeks	F.	++	++	++	Diffuse	501	
18 weeks	F.	++	++	++	Central	364	6.6
18 weeks	M.	±	+−−	±	Central	71	9.6
18 weeks	M.	++	++	±	Diffuse	274	7.3
23 weeks	F.	+	±	+	Diffuse	211	6.5
23 weeks	M.	±	—	±	Central	80	8.7
30 weeks	F.	++	++	+	Diffuse	449	8.8
30 weeks	F.	+	+	±	Central	173	8.6
30 weeks	M.	±−−	—	±−−	Central	50	8.4

[1] The vitamin A fluorescence of an adult stock animal on diet 13 corresponds to 2 plus signs.

fluorescent droplets in the neighborhood of the vessels disappeared.

Livers containing from 700 to 4000 units revealed the picture of a normal liver rich in vitamin A in a homogenous distribution; the fluorescence of the liver cells was parallel or even greater than that of the Kupffer cells (figs. 3, 4) although it gradually diminished with advancing depletion. When the total amount dropped below 700 units the continuous row of fluorescent droplets on the edge of the liver cells disappeared and the droplets were only found in the vicinity of the Kupffer cells (figs. 5, 7). Again the Kupffer cells showed a preponderance of fluorescence which became definite when the total amount fell below 250 units (fig. 6). The homogenous distribution disappeared; some part of the lobules, usually the periphery, was first depleted. Gradually the dim fluorescence of the cytoplasm of the liver cells disappeared. The last remnants of the vitamin A fluorescence were imparted by a few Kupffer cells in the central part of the lobules (fig. 8). In the periportal fields some ultra violet, stable brown or yellow inclusions became visible. The fat distribution followed that of vitamin A rather closely.

In the parallel study of Brenner, Brookes and Roberts ('42) based on the chemical assay a definite sex difference has been established. The amount of vitamin A in the liver after controlled feeding is higher in female rats and during depletion the male rats lose vitamin A much faster than the females. The first point was not substantiated histologically because no reliable differences were recognized in the higher ranges; the greater speed of depletion in males was histologically quite apparent. All the stages described above were reached faster by the male than by the female rats. In the males the preponderance of the fluorescence of the Kupffer cells disappeared and reappeared more rapidly than in females, in accordance with the faster drop of the total amount (compare fig. 4 with fig. 5, and fig. 6 with fig. 7). Complete lack of vitamin A fluorescence was reached neither in the males nor

females, but the fluorescence in the males at the end of 30 weeks was much lower than was that of the females.

The serum vitamin A level did not show any relation to the total amount of liver fluorescence or the fluorescence of the Kupffer or liver cells. In the males in which the Kupffer cell fluorescence was lost more quickly than in the females the blood level was higher, but occasionally a high blood level was associated with high Kupffer cell fluorescence in either sex. In the lower blood ranges especially, various degrees of Kupffer cell fluorescence were encountered, indicating the independence of the blood level from the Kupffer cell vitamin A.

The results of the eye studies agreed with the findings of Greenberg and Popper ('41). In the retina of dark adapted hypervitaminotic rats no vitamin A fluorescence was found. In light adapted eyes the fluorescence was found in high degree in the pigment coat, and in lesser degree in the rod and cone layer. In the former it was seen imparted by fine granules lining the hexagon shaped cells. Independent of the state of light adaptation the fluorescence was seen in the connective tissue cells of the ciliary processes in animals only after short depletion, but disappeared when the vitamin A in the liver dropped below 6000 units. The fluorescence of the retina did not entirely disappear, but seemed to diminish somewhat after a depletion period of 18 weeks.

DISCUSSION

The histologic and chemical methods agree in the estimation of the relative amounts of vitamin A present in the liver, except in the highest ranges where quantitative differences in the excess of fluorescence are not recognized histologically. This agreement is an additional evidence for the value and specificity of the histologic method. Furthermore, it permits the substitution of a much simpler histologic method for the chemical assay if a rough estimation of the vitamin A content of the liver is desired; in addition information on the localization of vitamin A is obtained. The histologic method is far superior if only parts of the structures of an organ

contain vitamin A. The chemical assay may not demonstrate this when these structures are buried in the great mass of vitamin A free tissue.

The importance of the localization is shown by the histologic studies. In the phase of high vitamin content there is a characteristic preponderance of the vitamin A storage in the Kupffer cells. In the normal stage relatively more is found in the liver cells than in the Kupffer cells. Whether an increase in the liver cells actually occurs, is not clear from the histologic picture. A decrease of the liver cell fluorescence, however, in the early stage of depletion of the animals which have received large amounts of the vitamin can be excluded. Our studies confirm the rapid loss of the vitamin A depots in the first 4 weeks (Davies and Moore, '35) with the chemical method, and less clearly with the histologic method, when the total amount of fluorescence is considered; however, the decrease found by us is somewhat slower. The histologic picture indicates that this rapid loss of the vitamin A stores of the liver concerns only the depots of the Kupffer cells. There is no relation between the blood levels at this time and the Kupffer cell fluorescence. Thus, there is no evidence that the Kupffer cells discharge the excess of vitamin A into the blood and we may assume that the Kupffer cells destroy this excess, thereby explaining the rapid loss.

Moore connected this non-economic utilization with a vitamin E deficiency of the rats since vitamin E seems to influence vitamin A storage in the liver (Moore, '40; Bacharach, '40). The possibility that vitamin E or its absence may be related to the destruction of vitamin A in the Kupffer cells could be considered. In our rats, however, there is no evidence for a vitamin E deficiency from the food intake, at least not in the early stages of depletion.

In the long middle period of depletion when a normal vitamin A store is present, there is preponderance of the fluorescence in the liver cells. Gradually their fluorescence decreases; this points to the liver cells as the physiologic stores for vitamin A. In advanced depletion a preponderance of

the Kupffer cell fluorescence reappears. The remnants of the vitamin A fluorescence in the liver cells are imparted by a few lipoid droplets adjacent to the Kupffer cells; previously, the entire edge of the liver cells had been lined by such droplets. Finally, only the Kupffer cells reveal the fluorescence. Apparently the Kupffer cells are concerned with the transmission of the vitamin A from the liver cells to the blood.

The lack of parallelism between the blood vitamin A level and the vitamin A fluorescence of the liver (which holds also for the amount of vitamin A chemically found in the liver), at least under the conditions studied, should be stressed. It coincides with the chemical analyses of Brenner, Brookes and Roberts ('42) who reported in greater detail the relation of the blood level to the liver stores and discussed the significance of their values. Blood levels of somewhat below 10 units are found in rats whose liver vitamin A fluorescence varies from +++ to the nearly deficient ±——.

As is already known (Greenberg and Popper, '41), the vitamin A fluorescence of the rat's retina depends much more on the state of light adaptation than on the nutritional state of the animal. No relation between the vitamin A fluorescence of the retina and the liver vitamin A stores or the blood levels was found, at least by the use of the histologic method which may conceal slight differences. Only after the eighteenth week was there apparently a slight decrease of the vitamin A fluorescence of the retina.

The female rats lose their vitamin A stores slower than the males, as is apparent from the chemical assays and histologic pictures. This sex difference is clearly shown if, as in this experiment, a uniform vitamin A supply has been administered during the feeding periods. The type of histologic picture seen during depletion is the same for the two sexes. Since in male rats a lower level is reached faster, the histologic changes occur more rapidly also. The higher blood level in male rats (Brenner et al., '42) cannot thus be explained by the histologic distribution in the liver.

SUMMARY

1. The histologic distribution of the vitamin A fluorescence in the liver was compared with the amount of vitamin A found chemically in liver and blood serum of rats first given large doses of vitamin A, and then placed on a vitamin A free diet. The vitamin A fluorescence ran parallel with the amount of vitamin A found chemically in the liver in all ranges of vitamin A content except when the largest amounts of vitamin were present where the differentiation in the degrees of fluorescence was less clear. This gave additional evidence for the value of the histologic method and recommended it as a simple technic for the estimation of the vitamin A content of organs. No simple parallelism existed between the vitamin A fluorescence of the liver and that of the retina or the blood vitamin A level.

2. Under conditions of large vitamin supply and advanced depletion there was a preponderance of vitamin A in the Kupffer cells over the liver cells. In the middle phase of the depletion period when the amount of vitamin A present compared favorably with that found in adult stock animals, much vitamin A was in the liver cells which probably represent the physiologic storage place. In hypervitaminosis the Kupffer cells store the excess of vitamin A and apparently destroy it; this may explain the uneconomic utilization of vitamin A under conditions of large supply. In depletion the Kupffer cells distribute the remnants of vitamin A.

3. During depletion the livers of male rats lose vitamin A faster than those of females. There is, however, no sex difference with respect to method of utilization, as judged from the histologic picture.

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PLATE 1

EXPLANATION OF FIGURES

Fluorescence photomicrographs of unstained frozen sections of livers, cut after short formaldehyde fixation and mounted in water. The livers are from rats which first received high vitamin A feeding and were then transferred to a vitamin A deficient diet. Magnification of 80. The results of the chemical assay of the liver for vitamin A in Evelyn Photoelectric Colorimeter units per 100 gm. liver as indicated in parentheses in legend are given for comparison (see table 1).

1 Female rat, no vitamin A deficient diet. Enormous vitamin A fluorescence imparted by the Kupffer cells, relatively little by fine lipoid droplets in the epithelial cells (12,931 units).

2 Male rat, 2 weeks on the deficient diet. Strong vitamin A fluorescence seen in the Kupffer cells, moderate in the epithelial cells (5525 units).

3 Female rat, 4 weeks on the deficient diet. Moderate vitamin A fluorescence of the Kupffer cells, relatively much fluorescence imparted by fine lipoid droplets in the epithelial cells (2562 units).

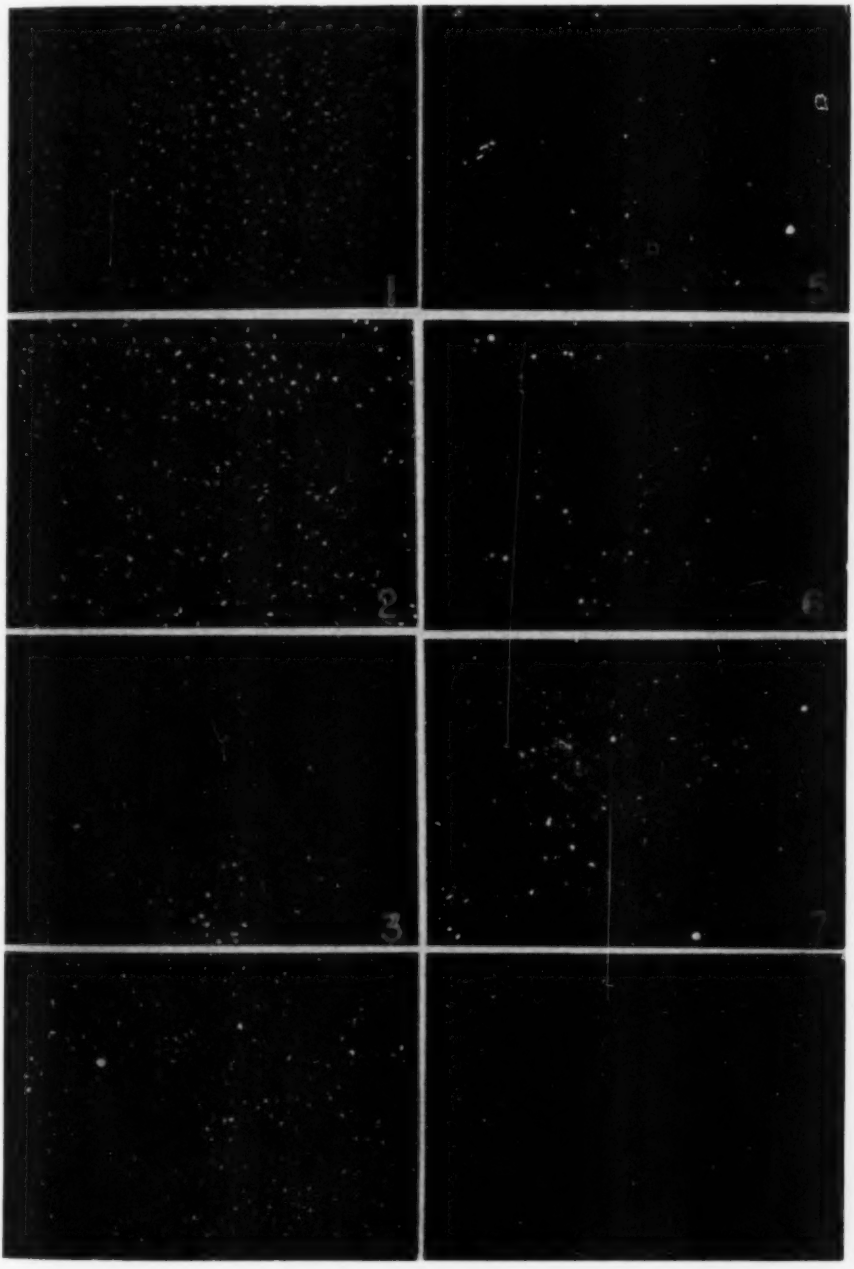
4 Female rat, 10 weeks on the deficient diet. Moderate vitamin A fluorescence of the Kupffer cells and of fine lipoid droplets in the liver cells, distributed close to the Kupffer cells. Homogenous distributions (965 units).

5 Male rat, 10 weeks on the deficient diet. Relatively little vitamin A fluorescence in Kupffer and epithelial cells. Inhomogenous distribution (350 units).

6 Male rat, 23 weeks on the deficient diet. Little vitamin A fluorescence of the Kupffer cells in irregular distribution (80 units).

7 Female rat, 30 weeks on the deficient diet. Moderate vitamin A fluorescence of Kupffer and epithelial cells (449 units).

8 Male rat, 30 weeks on the deficient diet. Traces of vitamin A fluorescence localized in scattered Kupffer cells (50 units).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EFFECT OF MANGANESE ON CALCIFICATION IN THE GROWING RAT

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TWO FIGURES

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Numerous experiments have been reported concerning the question of whether cations other than calcium can be deposited in the bones. Some of the results are of interest purely as experimental phenomena, indicating that the calcification mechanism is not absolutely specific for calcium salts; some, however, have shown the fate and to a large extent have guided the therapy of certain toxic metals in the body. Examples of metals used in such studies are lead, strontium, beryllium, radium, bismuth, and manganese.

Manganese, like beryllium, aluminum, and iron, has proved rachitogenic when fed in large amounts (Blumberg, Shelling and Jackson, '38). Since the major part of this element is excreted in the feces (Skinner and Peterson, '30), the harmful action is probably caused, not by the toxicity of manganese itself, but by its effect on phosphorus metabolism. Because appreciable amounts of manganese itself are absorbed and found in various tissues, including bone, there is also the possibility that it may have a direct influence on the calcifying mechanism.

Recent work has indicated that traces of manganese (25 to 50 p.p.m.) in the diet of chicks prevent perosis (Gallup and Norris, '39 a). A requirement as definite as that suggested for the chick has yet to be demonstrated in the rat, although manganese appears to have an important role in the produc-

tion and rearing of normal young of both rats and chickens (Gallup and Norris, '39 b; Orent and McCollum, '31; Barnes, Sperling and Maynard, '41).

The ingestion of certain acidic organic compounds has been reported to influence the cure of rickets by improving mineral absorption (Hathaway and Meyer, '39; Shohl, '37). Since manganese complexes with organic acids were observed by Delsal ('38), the effect of citrate on the mineral elimination in the feces was believed to constitute an important phase of any series of manganese metabolic studies.

The present investigation deals with three problems in manganese metabolism: (1) the effect of large manganese intakes on the fecal output of calcium and phosphorus and on the amount and location of the stored manganese; (2) the effect of phosphate, vitamin D, and citrate on both mineral excretion in the feces and the cure of rickets in the presence of manganese; (3) the probable role of very small manganese intakes in calcification.

EXPERIMENTAL

The experimental work with manganese in the diet was divided into two parts, the first dealing with high manganese intake and the second with small amounts of manganese.

High manganese levels

Young rats weighing between 45 and 50 gm. were depleted of their body stores of vitamin D by feeding some groups the Steenbock and Black ration no. 2965, and other groups the same ration with the calcium carbonate molecularly supplemented or replaced by manganous carbonate. The rachitogenic diets supplemented with manganese are designated as 1/5 S, 2/5 S, 3/5 S, and 5/5 S; while the rations in which the calcium was replaced by manganese are designated as 1/5 R, 2/5 R, 3/5 R, 5/5 R, 2/5 Rp, and 5/5 Ra. Diet 2/5 Rp had the phosphorus content increased by the addition of 1% potassium dihydrogen phosphate; in diet 5/5 Ra, 0.5% of the calcium carbonate was left in the diet (similar to diet C of Blumberg,

Shelling and Jackson, '38). The calcium, phosphorus, and manganese contents of these diets on a dry basis were as follows:

DIET	Ca %	P %	Mn %	DIET	Ca %	P %	Mn %
Steenbock	1.48	0.36	0.002	1/5 R	1.31	0.36	0.42
1/5 S	1.50	0.36	0.312	2/5 R	1.12	0.36	0.68
2/5 S	1.52	0.36	0.66	2/5 Rp	1.16	0.80	0.66
3/5 S	1.55	0.32	1.12	3/5 R	0.93	0.33	1.02
5/5 S	1.40	0.32	1.73	5/5 R	0.08	0.40	1.58
				5/5 Ra	0.84	0.51	1.22

All animals were placed in a darkened room in individual metal cages provided with raised screen floors. They were given ample rachitogenic diet and distilled water. A record was kept of food intake and change in body weight.

After a 21-day depletion period, half of the animals on each diet were killed by anesthesia in order to obtain blood and bones for analyses. The remaining animals were given (1) 5 units of vitamin D (U. S. P. XI reference cod liver oil), (2) 5 units of vitamin D with potassium citrate in the diet (0.4 mols per 100 gm.), or (3) 100 units of vitamin D. At the end of a 7-day curative period, these animals also were killed.

The blood samples obtained by cardiac puncture were pooled for each group of animals. Serum inorganic phosphorus was determined by the method of Youngberg and Youngberg ('30) and calcium by the Clark-Collip modification of the Tisdall method ('25). Livers were pooled for each group of animals. These were dried at 100° C., ground in a glass mortar, and ashed. The ash was analyzed for manganese by the periodate method of Willard and Greathouse ('17).

The feces were collected from individual animals during the 21-day depletion period and during the 7-day curative period. The dried fecal pellets were weighed and pulverized. Duplicate samples were ashed in platinum crucibles. The ash was dissolved in 5 ml. of nitric acid (1:4) by the addition of 1 to 2 ml. of hydrogen peroxide (3%) and the acid solution washed through a quantitative filter paper into a 100 ml. volumetric flask and diluted to volume with distilled water. Aliquots of this solution were used for analysis.

Phosphorus was determined by the volumetric method with ammonium molybdate precipitation (Hillebrand and Lundell, '29, p. 567) and calcium by permanganate titration of the oxalate in the presence of acetic acid (Ibid, p. 501). Manganese was determined by the periodate method, colorimetric measurement being made with an electrophotometer using a green filter with maximum absorption of 525 mμ.

Low manganese levels

The effect of small amounts of manganese on calcification was studied in the following experiments.

1. The Steenbock diet was supplemented with 0.005% and 0.01% manganese added as the carbonate.

2. The Steenbock diet was supplemented with tri-weekly subcutaneous injections of manganese lactate solution. The injections were made at levels of 2.5 mg. and 5.0 mg. per week; these amounts are equivalent to those in 50 gm. of diets containing 0.005 and 0.01% of manganese respectively. A level of 50 mg. per week was fatal 14 hours after the first injection. This high level was tolerated by adult rats, but their weight remained stationary, and after 2 weeks they were in very poor condition.

3. A non-rachitogenic, manganese-free, but otherwise adequate diet was used as a control. It consisted of whole milk powder [1] with supplements of 70 mg. of iron and 15 mg. of copper per week. No manganese could be detected in the ash of a 20-gm. sample of this milk powder by the method used. Manganese was added to the diet as a manganous sulfate solution containing 2.5 mg. manganese per milliliter. This was fed at levels of 1.25 and 2.5 mg. per week, representing 0.0025 and 0.005% dietary levels at a food intake of 50 gm. per week. The solutions of ferric chloride, copper sulfate, and manganous sulfate were mixed with 30 gm. of milk powder and dried. After the animals had consumed this mixture, they were allowed to eat the whole milk powder freely the remainder of the week. The groups were designated as MP, MP .0025, and MP .005.

[1] Klim.

The groups of young rats receiving these diets and supplements were subjected to the same procedures for the depletion and curative periods as described for the animals on high manganese levels with the exception of the rats in the milk powder groups. These animals received no vitamin D and were kept on the diet for 5 weeks.

RESULTS AND DISCUSSION

The average values for the results obtained from each group of animals are given in tables 1 and 2 and figures 1 and 2.

The growth response (table 1) indicated clearly the retarding effect of high manganese intake on growth in rats receiving the Steenbock diet. Considering the effect of manganese supplements with a high calcium-low phosphorus diet, it was evident that the growth of the animals decreased as the amount of manganese in the diet was increased (fig. 1). With equal amounts of calcium and manganese, the animals lost weight during the entire experimental period, losing about 30% of their initial weight in 4 weeks.

The condition of the animals may be explained by the abnormal calcium-phosphorus metabolism induced by the Steenbock diet itself and intensified by the presence of large amounts of manganese. The calcium excretion was increased with increased manganese intake (table 1). The phosphorus excretion was not only increased, but at the higher manganese levels, negative phosphorus balances were indicated by the fecal excretion of amounts of phosphorus greatly in excess of the dietary intake.

The replacement of calcium with manganese without increasing the actual mineral content of the diet produced a similar effect on growth and mineral absorption (table 1; fig. 2). Because a partial replacement of the calcium lowered the calcium content toward a more normal calcium-phosphorus ratio, the condition was less severe. The presence of manganese was still sufficient, however, to give a negative phosphorus balance.

TABLE 1

Average food intake, weight change, and mineral balance of the groups of rats receiving high manganese diets.

DIET	NO. OF RATS	FOOD INTAKE (DRY)	MINERAL INTAKE MG./RAT/DAY			VITAMIN D INTAKE	CHANGE IN WEIGHT	SERUM MG./100 ML.		BONE ASH	LIME TEST [2]	PER CENT INTAKE EXCRETED IN FECES			MANGANESE IN	
			Ca	P	Mn			Ca	P			Ca	P	Mn	Bone ash	Liver mg./100 gm. (dry)
21-day depletion period																
		gm.				*units*	*gm.*			%					%	
Steenbock	80	108	75.5	18.4	0.1		20.9	11.9	3.6	34.5		67.6	76.5	80.0		
1/5 S	24	95	70.5	16.9	14.6		9.3	11.6	4.1	36.5		77.6	83.5	95.9		
2/5 S	42	93	73.0	17.2	31.6		5.9	11.6	3.1	33.8		70.8	102.9	93.4		
3/5 S	25	85	71.3	14.7	51.5		1.6	11.0	3.5	36.9		72.9	125.2	68.9		
5/5 S	8	84	58.8	13.4	72.6		— 7.5		2.3	36.7		76.7	120.1	79.2		
1/5 R	9	93	59.0	16.2	18.8		11.1	13.4	2.6	39.3		69.5	108.6	81.9		
2/5 R	25	93	57.8	15.8	29.9		8.5	11.4	2.6	34.1		56.7	126.5	85.7		
2/5 Rp	4	109	60.2	41.5	34.3		17.3	10.8	3.7	33.3		66.7	61.2	55.6		
3/5 R	9	90	41.8	14.8	46.0		4.1		2.3	37.1		45.4	126.6	76.3		
5/5 R [1]	4	33	2.0	10.0	39.5		—10.0		4.6	44.5		1075.0	113.0	74.4		
5/5 Ra	6	63	24.4	14.8	45.9		— 9.6		2.9	39.8		59.0	85.2	81.2		
7-day curative period																
Steenbock	24	34	80.0	19.4	0.11	5	3.2	12.3	5.5	35.3	1.5	60.7	68.0	81.8	—	0.83
Steenbock	4	31	65.0	15.8	0.09	100	2.7	13.0	4.7	36.6	2.5	56.8	47.5	77.8		
Steenbock	4	44	90.3	21.9	0.14	5 + K Cit	3.3	10.0	6.1	36.0	3.5	35.4	26.4	78.5		
1/5 S	12	31	66.0	15.8	13.7	5	0.9	12.1	3.7	34.6	—0.3	57.2	74.0	86.1		
2/5 S	12	27	60.8	14.4	26.4	5	1.7	12.6	3.4	33.0	—0.8	59.8	84.0	72.4		
2/5 S	4	25	54.8	12.9	23.7	100	0.3		4.6	29.5	—1.8	72.9	104.7	86.1		
2/5 S	4	27	56.0	18.4	26.4	5 + K Cit	2.3		5.3	31.3	0.7	65.5	57.2	64.4		
3/5 S	8	26	62.0	12.8	44.8	5	— 2.1	12.6	3.2	34.8	—1.0	62.8	115.6	62.3	+	
5/5 S	4	22	43.4	9.9	33.7	5	— 6.0		3.3	31.7	—2.0	71.4	121.2	79.4	+	
1/5 R	5	34	63.0	17.2	20.2	5	3.4	12.2	4.5	33.4	—0.6	48.6	87.2	64.9	+	
2/5 R	12	26	40.4	13.0	24.5	5	— 0.3	12.2	4.1	31.3	—1.5	47.7	102.3	76.3	+	2.81
2/5 R	4	24	40.4	12.9	24.2	100	2.2	12.0	4.2	28.8	—1.3	65.7	89.9	67.7		
2/5 R	4	25	51.8	17.4	22.9	5 + K Cit	3.0	12.0	4.6	32.6	1.5	40.8	45.4	72.9		
2/5 Rp	4	39	63.9	44.0	36.2	5	3.0	11.6	6.0	36.8	2.5	34.3	45.9	65.4		1.71
3/5 R	6	27	37.0	13.2	40.9	5	— 2.8	13.0	3.8	34.5	—2.0	45.0	94.7	69.1	+	5.20
5/5 Ra	6	20	24.4	14.7	45.7	5	— 2.0	12.8	2.9	36.4	0.2	42.1	68.7	64.3	0.007	3.92
												5/5 R (for 14 days)				6.83

[1] Values given for 14 days instead of 21 days.

[2] Where a line test of (—1) indicated mild rickets, (—2) moderate rickets, (—3) severe rickets, and (—4) extreme rickets.

[2] Values given for 14 days instead of 21 days.
[3] Where a line test of (—1) indicated mild rickets, (—2) moderate rickets, (—3) severe rickets, and (—4) extreme rickets.

TABLE 2

Average food intake, weight change, and mineral balance of the groups of rats receiving low manganese diets.

DIET	NO. OF RATS	FOOD INTAKE (DRY)	MINERAL INTAKE MG./RAT/DAY			VITAMIN D INTAKE	CHANGE IN WEIGHT	SERUM MG./100 ML.		BONE ASH	LINE TEST [3]	PER CENT INTAKE EXCRETED IN FECES			MANGANESE IN	
			Ca	P	Mn			Ca	P			Ca	P	Mn	Bone ash	Liver mg./100 gm. (dry)
21-day depletion period																
		gm.				*units*	*gm.*			%					%	
Steenbock	80	108	75.5	18.4	0.10		20.9	11.9	3.6	34.5		67.6	76.5	80.0		
0.005% Mn	32	116	77.0	19.9	0.37		20.8	12.2	3.3	33.4		77.0	79.5	89.2		
0.01% Mn	48	113	74.5	21.6	0.68		19.2	11.8	3.4	37.1		70.6	67.2	102.9		
0.005% Inj. [1]	4	114	86.0	20.8	0.12		22.5					47.7	54.8	266.7		
0.01% Inj. [2]	6	97	45.2	15.8	0.09		12.2					72.7	83.6	455.6		
MP	4	119	62.8	55.8	....		54.1					49.3	30.5	....		
MP .0025	4	111	58.2	52.0	0.18		53.5					42.8	27.0	55.6		
MP .005	4	113	55.0	49.0	0.36		50.0					51.8	30.0	55.5		
7-day curative period																
Steenbock	24	34	80.0	19.4	0.11	5	3.2	12.3	5.5	35.3	1.5	60.7	68.0	81.8	....	0.83
0.005% Mn	12	38	73.5	18.9	0.35	5	5.4	12.6	5.1	33.5	1.5	62.0	66.7	88.6		0.89
0.01% Mn	16	36	67.7	19.6	0.56	5	4.9	12.0	5.7	36.5	1.5	65.5	63.2	100.0	....	0.99
0.005% Inj. [1]	4	27	54.8	13.3	0.07	5	2.0	13.0	4.0	34.4	—0.1	65.7	90.5	700.0	0.014	1.70
0.01% Inj. [2]	6	31	65.1	15.8	0.09	5	4.0	13.1	3.2	36.1	0.8	55.0	72.2	722.2		2.10
MP	4	56	89.0	79.5	....		21.0	12.2	10.4	56.6	4.0	52.8	27.3	....	....	0.28
MP .0025	4	55	86.8	77.4	0.18		24.0	12.6	10.7	55.8	4.0	54.7	33.1	61.2		0.42
MP .005	4	54	83.5	74.3	0.36		22.0	11.6	11.2	56.0	3.6	50.7	33.6	61.1	....	0.65

[1] Injections averaged 0.36 mg. manganese/rat/day.
[2] Injections averaged 0.71 mg. manganese/rat/day.
[3] Where a line test of (1) indicated just perceptible healing, (2) distinct healing, (3) advanced healing, and (4) normal bone or complete healing.

Addition of phosphate to the diet nullified the action of manganese on phosphorus excretion and improved the appetite and general condition of the animals (diet 2/5 Rp). It is notable, however, that excess of both calcium and phosphorus in this diet allowed a marked increase in manganese retention during the depletion period.

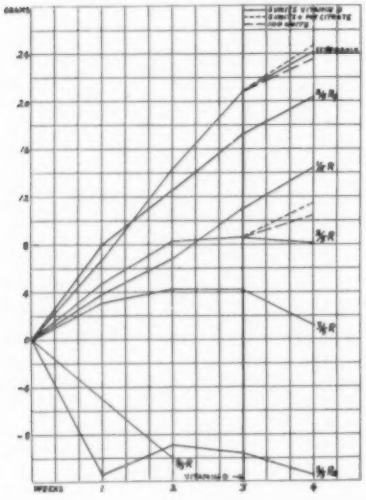

Fig. 1 Growth response in rats in the presence and absence of vitamin D when dietary calcium is supplemented with large amounts of manganese.

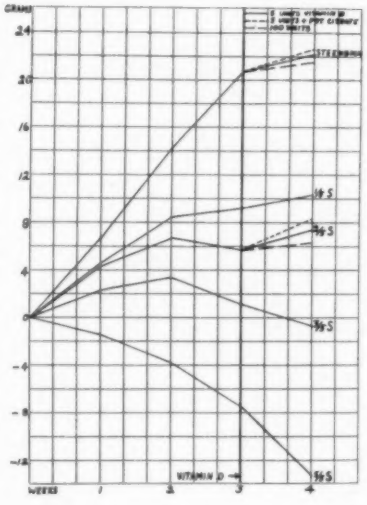

Fig. 2 Growth response in rats in the presence and absence of vitamin D when dietary calcium is partially or entirely replaced by manganese.

The complete replacement of calcium and manganese (diet 5/5 R) resulted in a serious upset in mineral metabolism. Within 14 days some of the animals had died; the others were in very poor condition. The rapid fecal excretion of calcium and phosphorus from the body supply was fatal. Calcium was excreted at an average rate of 21.5 mg. per day, when the intake was only 2 mg. per day. The phosphorus excretion was also greater than the dietary intake, and the serum phosphorus was high.

As a check on this condition, a second group of rats averaging 65 gm. initial weight were fed this diet (5/5 R). The larger animals tolerated the total replacement of calcium no better than the smaller ones. Consequently, 0.5% calcium carbonate was returned to the diet (5/5 Ra) at the end of the first week. The amount of calcium was sufficient to check the rapid loss in weight and to improve the general condition of the animals.

Except in this attempt to replace all of the calcium with manganese, the effect of manganese was more pronounced in relation to phosphorus retention than to that of calcium. Decreasing the calcium to change the calcium: phosphorus ratio also improved mineral assimilation in the presence of manganese. Even when the calcium: phosphorus ratio was reduced so that the diet was no longer rachitogenic, the presence of manganese still induced a rachitic condition.

The administration of vitamin D to animals receiving high manganese diets improved the condition of the animals on all manganese levels, but the effect was most pronounced where the calcium was replaced by manganese. The calcium and phosphorus elimination in the feces was definitely decreased in the presence of vitamin D, and in most cases there was a small gain in weight.

The most striking values in the fecal excretion data for the animals of both the control and the manganese groups are those following the addition of potassium citrate to the diet with the vitamin D supplement. In the animals of the control group and the 2.5 R group the calcium retention was markedly increased; the phosphorus retention was far more than doubled in all three of the groups receiving the citrate (control, 2/5 S, and 2/5 R). The values for phosphorus absorption are given on the assumption that all of the 0.35 to 0.50% phosphorus in the various diets was in available form. Lowe and Steenbock ('36) reported that of the 0.33% total phosphorus in the Steenbock diet only 6% is inorganic, and the rest is in a poorly assimilated form. Hence, the intake values given in these data are maximum. The controls show 23.5 to

32.0% retention of the total phosphorus present in the diet which, according to the work cited, should be about all of the phosphorus available for absorption. Yet with the addition of potassium citrate to the diet, it is notable that almost 75% of the total dietary phosphorus was apparently absorbed. Citrate did not have a marked effect on manganese retention.

Although manganese was evidently instrumental in increasing the fecal excretion of calcium and phosphorus, the feces did not contain all of the ingested manganese. The question arises, was part of the severe condition of the animals caused by the toxicity of the absorbed manganese or by some specific effect on calcium-phosphorus metabolism other than their excretion into the intestine? Marked storage of manganese occurred in the livers of these rats. The amount stored was in proportion to the intake and absorption. It was noted that, although increased phosphorus of the diet (2/5 Rp) resulted in considerable retention of manganese, the amount found in the liver was not as high as from a similar diet without the added phosphorus (2/5 R). The ash of the bones and kidneys contained manganese, but the quantities present in the available samples were too small to allow quantitative measurement by the method used.

In addition to a direct toxic effect of stored manganese, its presence in the tissues may affect the local calcification mechanism in the bone. In the instances of the rats receiving the high manganese diets, both the low blood phosphorus and the results of the line tests indicated a condition of severe rickets. These values, however, are readily explained by the interference with calcium and phosphorus assimilation. With the levels of manganese in the diet that caused a negative phosphorus balance, the phosphorus was drained from both bones and other tissues, and the rachitic condition was probably complicated with osteoporosis. This is especially true with the diet 5/5 R where all of the calcium was replaced with manganese. After only 14 days on this diet, the skeletons of the animals were brittle shells of cortical bone. The epiphyseal line was regular and did not indicate the usual rachitic con-

dition, but the bone had been dissolved from the interior and the marrow cavity filled with blood. The mesothelial linings were hemorrhagic, and the muscles were soft and small. These conditions were undoubtedly caused by the draining of calcium and phosphorus into the intestine. There was nothing which can be interpreted as a specific manganese effect, for the same result could be obtained by maladjustment of the calcium and phosphorus intake alone.

A further indication that the condition was a result of excessive mineral excretion was demonstrated by its improvement with the administration of vitamin D. The general effect of vitamin D was to increase calcium and phosphorus retention and the serum phosphorus level. This increase in phosphorus retention was small but consistent, in spite of the fact that the manganese present was antagonistic to the increased absorption induced by vitamin D. The bones from several groups of animals fed manganese showed some signs of healing along the epiphyseal line, although there was no deposition in the decalcified area of the metaphysis as in normal healing.

Here again the potassium citrate supplement with vitamin D gave significant results as was expected from the improvement in growth and in calcium and phosphorus retention. With the citrate the blood phosphorus was increased and the bones healed to an extent greater than that caused by 100 units of vitamin D alone.

The effects of small amounts of manganese in the diet were not so outstanding (table 2). When added to the milk powder diet, manganese at levels of 25 and 50 p.p.m. showed no appreciable or constant effect on growth, calcium and phosphorus absorption, or blood and bone content. Under these conditions, an average of about 40% of the ingested manganese was apparently absorbed compared to 25% absorption from high manganese intakes when fed with the Steenbock diet. Liver data indicated storage of manganese in proportion to the level in the diet, but the livers of the rats receiving the manganese-free milk powder diet still contained appreciable manganese after 5 weeks' experimental period. About 70% of the total

phosphorus was retained compared to 25 to 30% from the Steenbock diet. The blood and bone analyses yielded normal values.

The groups of rats receiving 50 p.p.m. manganese either by injection or in the diet in combination with the Steenbock diet showed a growth rate, slightly but consistently above that of the control animals. On the other hand, with a level of 100 p.p.m. in the diet, the animals showed slightly retarded growth rates compared to the controls. With the exception of a higher food intake for the animals on the 0.005% levels, the calcium and phosphorus retention and blood and bone analyses showed no appreciable differences between the animals receiving manganese and the control group (table 2).

The injection of manganese in an amount equivalent to 100 p.p.m. in the diet retarded the growth rate to a more marked extent. It may also be seen that the phosphorus excretion was increased in these rats. The explanation for the increased fecal excretion of phosphorus may be in the excretion of the injected manganese, in part at least, into the intestine. The fecal excretion of manganese for both groups in which the manganese was injected was several times the amount ingested in the diet. This agrees with reports in the literature on the excretion of manganese in the bile (Reiman and Minot, '20). In the present studies the amount of manganese found in the livers of the animals injected was twice as great as when the same amount was ingested in the diet.

The injected manganese showed little detrimental effect on the healing of the rachitic condition developed on the Steenbock diet. Vitamin D induced healing in all cases. Even with the manganese injections, the bones of two out of the four animals on the 0.005% level and five out of the six on the 0.01% level showed healing. The serum phosphorus of the latter group, however, remained at a rachitic level consistent with the decreased retention of phosphorus in this group.

From the results of the subcutaneous injections of manganese it appears that the rat does not tolerate more than about 50 p.p.m. of manganese in the diet. At levels of 100 p.p.m. the detrimental effect became evident.

SUMMARY

The effects of various amounts of manganese in the diet of young growing rats were studied with special reference to the influence of this element on calcium and phosphorus metabolism in the presence and absence of vitamin D. The following conclusions were drawn from the data obtained.

1. On high manganese intakes the growth of rats was retarded in proportion to the amount of manganese in the diet.

2. High manganese intake in connection with high calcium-low phosphorus diets increased the excretion of phosphorus in all cases and of calcium at the highest manganese levels. This excretion was by way of the feces and in some cases was increased to the extent of depleting the body supply of these elements. The interference with phosphorus retention was more pronounced than was that with calcium retention.

3. The adverse nutritional condition was less marked when the calcium in the diet was replaced than when it was supplemented by manganese. When the calcium of the diet was completely replaced by manganese, the results were fatal because of the severe loss of calcium and phosphorus from the body.

4. A more favorable calcium: phosphorus ratio in the diet, produced by increasing the phosphorus content, tended to improve retention of these elements even in the presence of manganese.

5. Animals from these groups which had been fed large amounts of manganese developed severe rickets as evidenced by blood phosphorus level and by the condition of the bones. Vitamin D administration resulted in an improved condition.

6. The addition of potassium citrate to the diet produced a striking improvement in calcium and phosphorus retention and subsequently in bone calcification.

7. Manganese was stored primarily in the liver. That which entered the body by subcutaneous injection was stored to some extent in the liver and the bones, but the larger portion was excreted in the feces.

8. Low manganese intakes, comparable to those found effective in correcting perosis in the chick, did not retard

growth and showed little or no effect on bone calcification either in conjunction with normal or with rachitogenic diets.

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THE RELATION OF LIVER STORES TO THE OCCURRENCE OF EARLY SIGNS OF VITAMIN A DEFICIENCY IN THE WHITE RAT [1]

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ONE FIGURE

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Symptoms which have been suggested as evidences of subclinical vitamin A deficiency are nightblindness, lowered vitamin A in the blood, and dryness and roughness of the skin with eruption of papules. Although it is generally recognized that each of these occurs in advanced stages of vitamin A deficiency, there is no agreement as to their relative value in detecting the earliest signs of deficiency.

Since the liver is the body's chief depot for vitamin A, it has been assumed that a certain amount of this vitamin in the liver is necessary to prevent the appearance of these signs. If this is true, a study of the liver stores in relation to the order of occurrence of these several signs would be one approach to the problem of what constitutes the earliest indication of hypovitaminosis A. Since the liver assay is impossible on human beings, it was decided to resort to animal experimentation to throw light on this aspect of the problem.

EXPERIMENTAL

Two series of investigations were undertaken.

Series 1. A study was made of the appearance of the early signs of deficiency in relation to liver stores in 4-week old

[1] Submitted by Sadie Brenner in partial fulfillment for the degree of Doctor of Philosophy in Home Economics at the University of Chicago.

rats. Nine litters (four to eight rats per litter) from mothers fed Sherman's diet 13 were used. Two rats from each litter (one male and one female) served as positive controls. All the rats were fed the vitamin A-free diet [2]. In addition, the controls received butterfat and cod liver oil which were mixed with the diet, the mixture yielding 38 I. U. of vitamin A per gram of food. Individual records of weekly weights and food consumption were kept. One litter was sacrificed each week. Before anesthetizing the animal, the eyes were bleached for 10 minutes by exposure to bright light to insure a maximum concentration of vitamin A derived from the action of light on visual purple (Wald, '35). The body of the animal was then opened, the blood collected, and the liver and eyes removed. It was usually necessary to pool the blood of several animals in order to supply enough for one determination. The whole eyes of all the experimental animals in a litter were pooled and assayed for vitamin A. The livers were analyzed individually. The same procedure was used for the controls. The extracted blood and tissues were analyzed according to the methods of McCoord and Luce-Clausen ('34) and McCoord ('40), using the Evelyn Photoelectric Colorimeter. The macro unit was used for the livers and the micro unit for the blood and retinas. Analysis for carotene was not necessary because previous studies had shown it to be absent from the body of the rats on the diets used. Sections of skin were sent to the dermatology department for histological examination. Measurements of dark adaptation of the animals were attempted but proved to be unsatisfactory due to the lack of a tropistic response of rats to light.

Results of series 1. As shown in table 1, the livers of rats at weaning contained small but measurable amounts of vitamin A (27 units per 100 gm. of liver)[3]. These were entirely lost,

[2] The vitamin A-free diet consisted of 18% alcohol-extracted casein, 4% Osborne and Mendel salt mixture, 1% iodized salt, 67% cornstarch and 10% dried yeast (contributed by the Northwestern Yeast Company, Chicago, Illinois).

[3] Evelyn Photoelectric Colorimeter Units. All liver, eye and blood values refer to these units per 100 gm. of liver and eye tissue and per 100 cc. of serum, respectively, unless otherwise stated.

however, at the end of 1 week of depletion. The livers of the controls, on the other hand, increased steadily in vitamin A.

The blood and eye tissues, however, still contained appreciable amounts of vitamin A even after 7 weeks of depletion. At this time the litter examined had a blood value of approxi-

TABLE 1

The effects of depletion on the vitamin A content of the livers, blood and eyes, and on growth.

WEEKS ON DIET	NO. OF RATS		LIVER		BLOOD [1]		EYES [2]		WEIGHT	
	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control
	gm.	*gm.*	*units/100 gm.*		*units/100 cc. serum*		*units/100 gm.*		*gm.*	*gm.*
0 [3]	4	4	27	27	8.5	8.5	49	49	40	40
1	5	2	0	236	..	..	107	175	61	59
2	5	2	0	242	12.4	..	68	78	65	91
3	4	2	0	789	10.1	..	95	56	70	118
4	6	2	0	786	9.6	..	36	88	74	143
5	5	2	0	947	7.2	8.6	32	56	75	157
7 [4]	6	6	0	1149	4.7	15.5	44	93	78	199

[1] Blanks indicate results which were rejected due to the formation of a cloudy solution when $SbCl_3$ reagent was added to the evaporated petroleum ether residue.

[2] Two things must be borne in mind in interpreting the data obtained on the vitamin A content of the eyes: (a) the small amount of biological material available; (b) the dependence of the vitamin A content of the eye upon its light adaptation (Wald, '35). The number of eyes used for a single determination was at least four. In order to have a sufficiently concentrated extract of the eye tissues little solvent was used for grinding and washing; consequently the extraction of the tissues may not have been complete. Although the animals in this experiment were placed in the bleaching box for 10 minutes prior to their killing, this does not insure that all the animals were bleached to the same extent due to their varied activity. With these considerations in mind it is believed that the actual figures presented for the eyes can only be used to indicate trends or relative changes.

[3] One litter was used for the analyses of the experimental and control groups at zero weeks on the diet.

[4] Three litters of rats were sacrificed at this time.

mately one-half (4.7 vs. 8.5 units) and an eye content only slightly below that found in the first litter sacrificed (44 vs. 49 units). The blood and eyes of the litter mate controls at 7 weeks showed considerable increases in vitamin A above that found in the 4-week-old rats.

The weight gains of the experimental animals were slight in comparison with those of the controls. The deficient rats continued to gain up to the fifth week of depletion at which time a growth plateau was reached.

A microscopic study of the skin sections [4] revealed no difference in either the histological structure of the stratum corneum or the cutaneous glands of the control and experimental animals. The external appearance of the fur was also not markedly different in the two groups of animals. Even in one litter of rats allowed to die of vitamin A deficiency there were no measurable changes in the histological structures of the skin.

The signs of avitaminosis A which were present in the depleted animals were mild. The rats were small in size as compared to the controls; some of them exhibited a thinning of the fur on the head and slight inflammation and crustiness of the eyelids. Few rats showed difficulty in walking. Only one death occurred within 7 weeks, and that on the thirty-first day of depletion.

This experiment, then, showed that in the absence of liver reserves the rat is able to maintain appreciable amounts of vitamin A in the blood and eye, and to make slight gains in weight for several weeks. Due to the rapid loss of the liver stores, however, the purpose of this experiment, namely, to study the relation of liver stores to the order of the appearance of the early signs of vitamin A deficiency, was unfulfilled. Therefore, a second investigation was undertaken in which 4-week-old rats were made hypervitaminotic with vitamin A concentrate and the effects of subsequent depletion studied.

Series 2. Ten litters of rats 28 to 34 days of age were maintained on their stock diet, and fed approximately 14,000 I. U. of vitamin A [5] daily for 35 days. The supplement was admin-

[4] Dr. S. W. Becker of the Department of Dermatology of Billings Hospital studied the skin sections which were placed in Bouin's fixative, cut from paraffin and stained with hemalum, erythrosine and saffron.

[5] A shark liver oil concentrate furnished by Vitamins Inc., Chicago, Illinois, and halibut liver oil contributed by Mead Johnson and Company, Evansville, Indiana, were used.

istered orally from a calibrated dropper. Two comparable animals received an equal volume of cottonseed oil for the same length of time.

One litter, containing two males and two females, was sacrificed 24 hours following administration of the last dose in order to find the storage resulting from massive feeding. The other nine litters were placed on the vitamin A-free diet. One of these, also containing two males and two females, was sacrificed after 3 days of depletion to determine the rapidity in the rate of loss of liver stores. The remaining eight litters were then classified into twelve groups. Each group, except the last two, contained two males and two females. The basis of selection was such that the average age, weight, and representation from the different litters in the groups were as alike as possible. One group was sacrificed each week for the first 6 weeks of depletion; the time interval between observations was then lengthened in accordance with the rates of depletion. The entire depletion period lasted for 30 weeks, during which time fifty-three animals were examined. Chemical analyses for vitamin A were made of the liver, blood and eyes [6] as in the earlier series. In addition histological examinations of the livers and retinas were made by fluorescence microscopy (Popper and Brenner, unpublished).

Results of series 2. As shown in table 2, the average vitamin A content of the livers and blood of the animals sacrificed 24 hours following the cessation of massive feeding was large in comparison with that found in the stock animals which received cottonseed oil. Throughout the first week of depletion the liver content of the animals sacrificed showed no reduction [7]. Thereafter the liver stores decreased rapidly. In the males 63% of the original stores was lost by the end of the second week, and 93% by the eighth week. After that the loss was more gradual, 98% of the original stores being lost only

[6] These eyes were bleached for 30 minutes.

[7] Since the storage ability of the individual animals varied somewhat, an average of the liver values for the first three groups was used to express the amount of vitamin A storage resulting from massive feeding. The calculations which follow are based on this figure.

at the end of the eighteenth week. The amount remaining in the liver was representative of that found in adult stock animals. The results for the females were similar except that they had a higher vitamin A liver content than did the corresponding males in all groups but one.

The effects of depletion on the blood were more immediate than on the liver stores. Within 3 days the blood level had

TABLE 2

The effects of depletion on the average vitamin A content of the livers, blood and eyes of hypervitaminotic rats[1]

DEPLETION TIME	WEIGHT		LIVER WEIGHT		VITAMIN A CONTENT						EYES
					Livers				Blood[2]		
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
	gm.	*gm.*	*gm.*	*gm.*	*units/100 gm.*		*units/total liver*		*units/100 cc. serum*		*units/100 gm.*
0 days[3] cotton-seed gp.		164		6.485		252		16		10.5	
vit. A gp.	191	135	8.796	6.753	9,133	12,340	794	833	40.0		
3 days	185	139	9.290	6.407	7,688	15,649	764	1003	18.7		
7 days	201	163	8.398	6.791	13,078	17,096	1105	1222	18.7		
Average	192	146	8.828	6.650	9,966	15,028	888	1026			
2 wks.	194	155	8.491	6.351	3,663	6,103	303	388	13.2	11.5	83
3 wks.	278	168	10.625	6.230	5,088	4,419	534	276	19.8	9.4	
4 wks.	246	167	8.941	5.962	1,669	3,750	218	150	14.7	8.8	70
5 wks.	251	196	8.619	6.539	1,836	3,783	162	247	17.0	13.1	69
6 wks.	255	173	8.446	5.908	849	3,248	72	182	13.5	7.7	98
8 wks.	314	190	10.089	5.593	684	1,883	69	106	12.4	8.8	35
10 wks.	299	191	9.697	5.418	294	1,115	29	61	15.4	8.6	27
13 wks.	256	181	6.864	5.213	170	1,661	12	85	10.5	6.5	28
18 wks.	272	179	7.366	5.532	159	433	12	24	8.4	6.6	40
23 wks.	270	202	6.926	6.778	80	211	6	14	8.7	6.5	28
30 wks.	302	191	8.028	5.801	50	311	4	20	8.4	8.7	36

[1] Each figure represents the average for the two animals of the same sex except in the last two groups where there are one male and one female, and one male and two females, respectively.

[2] In the first three groups the blood samples of the males and females were pooled so that no separate values are available for the sexes for these periods.

[3] Determinations were made 24 hours after the last dose.

dropped from its initial value of 40 to 18.7 units. Thereafter it continued to fall, but at a much slower rate, until a plateau was reached at about the thirteenth week. If the first high blood value is discounted on the grounds of incomplete removal of vitamin A by the liver, then the average blood level is seen to have decreased from 18.7 to 8.4 units for the males at the thirtieth week of depletion. The average liver stores for that same period dropped from 9,966 to 50 units. The findings

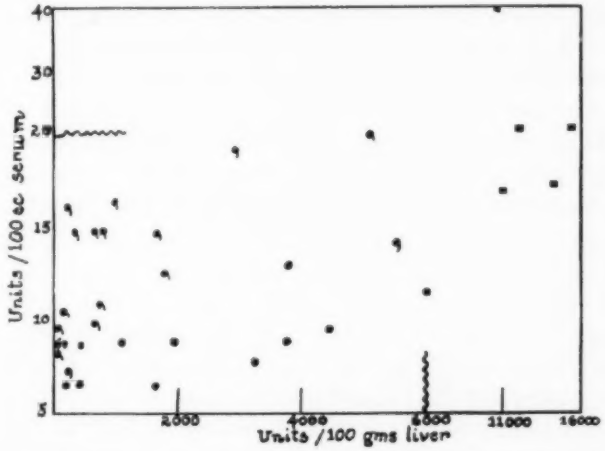

Fig. 1 The relation of blood level to liver stores. Blood values of the males in most cases represent unpooled blood and are plotted against their corresponding liver values; the blood values of the females are usually from the pooled blood of the two females in the group and therefore plotted against their average liver stores. Tagged dots, males; Untagged dots, females; Squares, pooled male and female values.

in the females were again similar but in practically all cases the males had higher blood levels than did the corresponding females.

To determine more clearly the relation between liver stores and blood values, the liver and corresponding blood values for each sex were plotted (fig. 1). A slight tendency toward higher blood values with increasing liver stores during the

early periods of depletion was revealed, especially in the males. However, any one particular blood level covered a wide range in liver stores and, conversely, one liver value was accompanied by a wide range in blood level. For example, a blood level of 8 to 9 units was found with liver stores from 50 to 3,750 units, while a liver storage of approximately 500 units was associated with blood values of 6.5 to 14.9 units.

The vitamin A content of the retina was 83 units at the end of the second week of depletion. At the eighth week there was a decided decrease to 35 units. This approximate level was maintained for the duration of the experiment. A similar amount was found in the eyes of the rats of series 1 after 7 weeks of deficiency.

In this series, the following definite sex differences were observed in respect to storage of vitamin A, rate of depletion and blood levels: (1) On equal intakes, the females had a greater capacity for storage, the males storing two-thirds the amount of the females on the 100 gm. basis. Similar results are obtained on comparing the content of the total livers, though here the difference is not as marked because of the larger livers of the males. (2) The females not only stored more vitamin A, but also retained it more tenaciously (table 2). At the end of the sixth week the males had lost 91% and the females 79% of their original stores; by the end of the thirteenth week the losses were 98% and 91% respectively. At the thirtieth week the livers of the females were found to contain 311 units per 100 gm.; those of the males only 50 units, or approximately twice that in stock rats at weaning. (3) In keeping with their greater capacity for storage and greater ability to retain their stores, the females also had lower blood levels than the males, suggesting a more conservative utilization of vitamin A by the various organs supplied by the blood. To determine the significance of these findings Fisher's t-test was used and the difference found to be significant; these sex differences would have occurred by chance less than one time out of one hundred.

DISCUSSION

Liver stores in 28 day-old rats of diet 13 stock animals disappeared within 1 week whereas the stores of hypervitaminotic rats were still partially filled after 30 weeks on a depletion diet. These rapid losses in young rats have not heretofore been reported. Horton et al. ('41) found almost complete loss in 3 weeks, and Popper and Greenberg ('41) in 2 to 3 weeks depending on the weaning age. Such discrepancies may be explained partly on the basis of the varying nutritional history of the rats and on the different methods used for analyses of liver stores. The rapidity of loss of the excess liver stores of the hypervitaminotic animals confirms the early work of Davies and Moore ('35). The rates of loss in their study were identical with those found in this study through the eighth week of depletion; from then on the rate was slower in the present study. Moore ('40), in later work, failed to confirm the rat's lack of economy in utilizing its excess stores. He attributed the rapid loss obtained previously to a lack of vitamin E in the depletion diet since vitamin E seems to influence the vitamin A storage in the liver (Moore, '40; Bacharach, '40). In the present experiment the animals were kept on diet 13 for the length of the entire supplementary feeding period and it would be expected that enough vitamin E was stored so that the early phase of depletion was not affected.

Although the vitamin A content of the blood and eyes of hypervitaminotic rats decreased from their original values during the process of depletion, the ability of the organism to maintain significant amounts of vitamin A in these tissues in spite of continued absence of liver stores was demonstrated over a period of several weeks. This confirms quantitatively the work on the retina by Greenberg and Popper ('41) who used fluorescence microscopy. In the light of these findings one may question the value of dark adaptation as an indication of one of the early signs of vitamin A deficiency.

The persistence of vitamin A in the blood and eyes, in addition to the small gains in weight made by the animals with no liver stores, suggests the possible presence of vitamin A

in a usable form in other body tissues, thus helping to keep vitamin A in the blood and eye during depletion until these "emergency" stores are used up. This agrees well with Dann's work ('32, '34) which showed that there was no correlation between the vitamin A liver stores present at weaning and the survival time. He suggested a tissue vitamin A not as yet chemically detected. Popper and Greenberg ('41) have demonstrated that the kidneys and adrenals are the only organs other than the retina which retained vitamin A in the absence of liver stores. Horton et al. ('41) did not find vitamin A in the blood when liver stores were empty.

In this study the blood level was found to be of slight value as an index to the nutritional status of the rat except perhaps in extreme deficiency and immediately after massive feeding. These states of vitamin A nutrition were accompanied, respectively, by extremely low and high blood levels. The diagnostic value of intermediate blood levels was by no means clear-cut. All that could be concluded was that higher blood values than those obtained in extreme deficiency, although they tended to be somewhat proportional to liver storage at the upper ranges, can only be interpreted as meaning that stores are present in other parts of the body (i.e., kidney and adrenals) and not as indicating the quantity of reserves in the liver. Closer relationships were obtained between the blood level and liver stores by Horton et al. ('41) and Lewis, Bodansky et al. ('41). A possible cause of these discrepancies is found in Leong's work ('41) which showed that the type of relationship depended upon whether the graded feeding or the depletion technique was used. Positive correlations were obtained with the former, no correlation with the latter.

Sex differences in the vitamin A content of the liver and blood and in the rate of loss of liver stores have previously been reported. From her analysis of the figures of Lagoras and Drummond on control animals, Kimble ('39) has pointed out a sex difference in favor of the females in the ability to store vitamin A. She has also found in human beings that males have a significantly higher blood level than females.

Murrill et al. ('41) have substantiated this conclusion. Bult and Sorgdrager ('38) found a slower rate of loss of vitamin A stores in female and castrated male rats than in normal males. The higher blood level and lower vitamin A stores of the rats may be taken as an indication of a more rapid dispersal of the liver stores, in contrast to the greater economy in utilization by the female. This is not surprising in the light of the role of the female in reproduction and lactation.

It would seem then from this study, that in the rat the use of the vitamin A content of the blood and retina or changes in the histological structure of the skin as early detectors of vitamin A deficiency is not warranted if by deficiency is meant the lack of liver stores. All three of these measurements were resistant to change even in the absence of liver stores. However, it was shown that, although a blood value for a particular rat could not be used to estimate the quantity of its liver stores, the blood value might have some diagnostic significance for large numbers of rats, where in general it could be assumed that higher blood levels were more apt to be associated with higher liver storage than were low blood levels.

SUMMARY

A study of the vitamin A liver stores in relation to the appearance of several alleged subclinical signs was undertaken in rats on a vitamin A-free diet. It was found that:

(1) Whereas 4-week-old rats lost their total vitamin A liver stores after 1 week, hypervitaminotic animals lost 90% of their excess stores after 8 weeks and 98% after 18 weeks. At the thirtieth week of depletion the liver still contained vitamin A.

(2) The vitamin A content of the blood declined rapidly from the hypervitaminotic level, and then decreased more gradually until a plateau was reached for both sexes at about the thirteenth week.

(3) The vitamin A content of the retina decreased to the eighth week of depletion when a plateau was reached.

(4) In the absence of liver stores for several weeks vitamin A was still present in the blood and eye in amounts found in stock rats of weaning age.

(5) A significant sex difference existed. The females stored and retained more vitamin A in the liver than the males, while the males had higher blood levels.

ACKNOWLEDGMENT

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FACTORS INFLUENCING THE ONSET AND CURE OF NUTRITIONAL MUSCULAR DYSTROPHY

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In an earlier paper (Eppstein and Morgulis, '41) we reported our findings, in agreement with those of Mattill ('40), demonstrating the ineffectiveness of dl-α-tocopheryl acetate for curing nutritional muscular dystrophy of rabbits when administered parenterally. The question was raised, whether this failure was due to the lack of an esterase within the body to liberate the free tocopherol, as suggested by Mattill ('38), or whether it was due to the slow rate of absorption of the oil solution of the vitamin, the animal succumbing to the disease before a sufficient amount of the vitamin becomes available to its tissues. In order to answer this question we have employed in this study a water-soluble derivative, disodium α-tocopheryl phosphate. Further, we endeavored to eliminate the factor of rancidity in the vitamin E-low diet by the use of non-oxidative procedures to remove the vitamin; and, finally, a new technique was devised for following the development of dystrophy by the urinary creatine excretion.

METHODS

As in our previous work young albino rabbits were employed. The vitamin E-deficient diet, XP, is a synthetic mixture containing the major foodstuffs, as well as minerals and roughage, in amounts approximating those found in commercial complete rabbit foods. Vitamin B complex was supplied by a rice bran concentrate,[1] and vitamin K by 2-methyl,

[1] Vitab, type II.

1, 4-naphthoquinone,[2] both these materials being incorporated directly in the food. Vitamins A and D were supplied by a weekly supplement, per os, of 0.1 ml. of a concentrated preparation.[3] Unsaturated fatty acids were furnished by feeding 1 ml. of linseed oil once a week, irradiated to destroy the vitamin E. For this purpose quartz flasks, about 1¼ inches in diameter, were filled with oil, tightly stoppered, and exposed to the sun (November) for at least 2 weeks. This was followed by irradiation for 6 hours at a distance of 1 inch from a Labarc Mercury vapor lamp.

The casein was extracted seven times with petroleum ether, as previously described (Eppstein and Morgulis, '41). The regenerated cellulose [4] was ground to a suitable fineness in a corn mill. Olive oil, rather than lard or "seed oils," was employed as the source of fat in order to keep down the vitamin E content. To the dry ingredients, thoroughly mixed, an emulsion of the rice bran concentrate [5] and olive oil was added together with sufficient water to make a stiff dough. This dough was then pressed through ¼ inch wire mesh, spread out and dried for about 15 hours at room temperature with the aid of an electric fan. There could have been only very little loss of the B vitamins since the pH of the dough was about 5.0. The pellets thus produced were pleasant to the taste and, although on exposure to air for several weeks no rancidity was detectable by taste or odor, the material was kept in a well-closed can. The diet was made up fresh every 5 days. Reared on this diet the rabbits seemed healthy but did not grow as well as on our other diets (table 1).

The disodium α-tocopheryl phosphate was prepared from natural α-tocopherol [6] essentially by the procedure of Karrer and Bussman ('40). The salt, dissolved in a minimum of water, was precipitated by the addition of 5 volumes of

[2] Kindly supplied by Mead Johnson Company.

[3] Oleum percomorphum 50% (Mead Johnson).

[4] Kindly supplied by the Sylvania Corporation.

[5] See footnote 1, page 473.

[6] We wish to thank Merck and Company for their generosity in furnishing the product.

acetone, then dried first with acetone and finally in vacuo at 80°C. over phosphoric anhydride. The resultant grayish white powder was only slightly hygroscopic. Analyzed for phosphate by the method of Fiske and Subbarow, as modified for the Evelyn photoelectric colorimeter, its P was found to be 5.51 and 5.62%. This may be compared with a calculated value of 5.59% for P in $Na_2PO_3C_{29}H_{49}O_2$. Inorganic phosphate was not detectable in a 4% solution.

The disodium salt of α-tocopheryl phosphate forms an opalescent foamy aqueous solution, which in a 0.944% concentration (equivalent to 5 mg. α-tocopherol per milliliter)

TABLE 1

Diet XP

Extracted casein	100.0
White dextrin	240.0
Regenerated cellulose	60.0
Sucrose	25.0
Salt mixture (Hawk and Oser)	20.0
Vitab, type II	50.0
Olive oil (containing 40 mg. of 2-methyl 1,4-naphthoquinone	20.0
Total	515.0

has a pH of 9.67 (glass electrode). A 5.5% solution of the monosodium salt (prepared by adding the theoretical amount of HCl) forms a white thixotropic gel which easily passes through a no. 26 hypodermic needle. This solution has a pH of 4.2, but does not taste acid, possibly because of its gel texture. The barium and calcium salts are insoluble in water. All three metallic salts form colloidal solutions (and gels) in fat solvents, such as ether or petroleum ether, but are insoluble in alcohol or acetone. The free acid is somewhat soluble in water but much less so when the solution is acidified. The free acid precipitates are stringy white solids.

The solution of disodium α-tocopheryl phosphate (equivalent to 5 mg. α-tocopherol per milliliter) was preserved with a crystal of thymol in a brown vaccine bottle and autoclaved

at 20 pounds pressure for 30 minutes. Intramuscular injection of this alkaline solution caused neither local reaction nor evidence of discomfort. Injections of 1 ml. were given to the dystrophic rabbit, the dose being repeated when the anticipated end of the protective period approached. The period of protection was counted from the time of the injection until creatinuria reappeared. The calculation of the minimal requirements was made as before in terms of milligrams per kilogram body weight per day.

The onset of dystrophy was evidenced by development of the usual clinical signs and in addition by the determination of the ratio of urinary creatine to total creatinine (preformed plus creatine). The finding of Morgulis and Spencer ('36) that dystrophic animals excrete large amounts of creatine during the active or "acute" phase of the disease has been utilized by Mackenzie and McCollum ('40) as a criterion of dystrophy by determining the absolute increase in creatine excreted per day. This procedure is tedious and involves the use of metabolism cages. As a test for the progress of dystrophy such elaboration is quite unnecessary, for, if the increase in creatine output is referable to a breakdown in muscle metabolism, the urine at any given time should reflect the existing muscular condition. The ratio of creatine to total chromogenic material in a given urine sample will disclose an existing creatinuria as readily as, and under certain conditions even better than, the absolute increase in a 24-hour period. The simplicity of this technique makes the test a practical adjunct in studying nutritional muscular dystrophy.

In practice, the test is carried out in the following manner. A sample of urine is expressed from the bladder and sufficiently diluted so that the chromogenic reaction can be accurately determined. Usually the required dilution can be easily estimated from the color of the urine itself. There is no need to measure either the amount of urine or the degree of dilution. Samples of this diluted urine are analyzed for preformed and total creatinine and the ratio: creatine $\times$ 100/ total creatinine, is calculated. The color determinations are

very easily carried out with an Evelyn photoelectric colorimeter, making a slight modification of the regular Folin procedure. Nitrogen digestion tubes calibrated at 50 ml. and capped with glass thimbles are most convenient both for carrying out the autoclaving and for the subsequent color development. Normal values of the creatine ratio vary from 0 to 10. With the onset of the acute phase of dystrophy this ratio jumps to 30 or 40 overnight, and to about 55 or 60 in another day and, finally, to 75 or 80. Unless treatment is instituted before the ratio reaches the value of 70–80 there is little likelihood of a subsequent cure. With adequate and prompt treatment of the diseased rabbits, the ratio falls rapidly and returns to the normal limits within 2 to 4 days.

EXPERIMENTAL

Effect of cod liver oil. Twenty-one rabbits were put on diet XP. Mild dystrophic symptoms occurred in the usual time of 3 to 5 weeks. Nevertheless the animals continued to grow and their dystrophy did not tend to become more pronounced. The creatine ratio remained essentially normal. Occasionally the growth curve would show a plateau or dip down for a few days while symptoms of dystrophy would also become more pronounced. Immediately prior to or during this period the creatine ratio often would rise to values above the normal. Some animals showed no change in the rate of growth at any time. When by the end of the fifth week no active dystrophy appeared, we suspected that perhaps rancidity was an essential contributing factor for its development. Accordingly, nine animals were given daily supplements of cod liver oil (approximately 1 ml. per kilogram body weight) instead of the weekly percomorph oil supplement. As can be seen from the data recorded in table 2 six of these animals became manifestly dystrophic within 6 to 10 days, the other three in 21 to 23 days. The rabbits which were not fed cod liver oil showed no change in condition. On the fifty-eighth day four more rabbits were given the cod liver oil supplement and all became actively dystrophic within 3 to 10 days. The remain-

ing eight animals on the original diet continued to grow, and, except for a slightly awkward gait and occasional spurt of creatinuria, appeared to be in good health. They were sacrificed on the seventy-eighth day when muscle samples were taken for histological study.

Microscopic examination of the sections showed a latent or "subacute" dystrophy varying from mild (comparatively few muscle fibers in a section affected) to severe (most of the fibers affected). The commonest finding was the loss of transverse striations. Reactive cellular regeneration was fairly common, and a mild fibrosis was also occasionally found.

Because the terms "acute" and "chronic," besides their use in a temporal sense, have in pathology acquired very definite meanings, not necessarily related in any way to time, an unfortunate confusion exists. In the present case the rabbits on the non-rancid diet had a "chronic" dystrophy from the temporal point of view, but pathologically most of the animals presented some state of "acute" dystrophy. In view of the established use of these terms by the pathologist it would perhaps be best to use a different system to designate the temporal phases. Thus the animals not receiving cod liver oil could be said to have a latent or cryptic dystrophy which develops into the clinically manifest or overt dystrophy when cod liver oil is given.

This response of the rabbits to the feeding of cod liver oil is in agreement with the report of Morris ('39) and the extended investigation of Mackenzie and McCollum ('40) although the degree of dystrophy found by the latter workers in the cod liver oil free rabbits was far more severe. The simplest explanation of the results would seem to be that the extracted diet still had sufficient vitamin E to prevent severe and general dystrophy but not enough to prevent gradual dystrophic changes, and therefore the clinical picture was not striking. The feeding of cod liver oil, as pointed out by Mattill ('38), allows the development of rancid products in the gut. This may have accomplished destruction of the

residual vitamin E, causing overt dystrophy to develop. However, there may be also an alternative interpretation of these results.

Parenteral administration of disodium α-tocopheryl phosphate. The α-tocopheryl phosphate ester is very effective in producing cures when injected intramuscularly into rabbits showing active dystrophy. Of the twelve animals so treated eight were rapidly cured. Three of the four failures were already in a very advanced stage of dystrophy, their urinary creatine ratios being over 70 before treatment was instituted. The fourth rabbit had a ratio of 60, but by the next day this rose to 71, then on the following day to 74, when the animal died.

The animals cured with α-tocopheryl phosphate showed minimum daily requirements varying from 0.20 mg. to 0.64 mg. per kilogram body weight. The average value is 0.31 mg., or 0.26 mg. if the single unusually high value of 0.64 mg. is not included in the calculation. Two comparative tests were made with orally administered sesame oil solutions of dl-α-tocopheryl acetate [7] equivalent to 10 mg. of tocopherol. One of the animals previously cured with the phosphate ester (minimum requirement 0.43 mg.) now showed a minimum requirement of 0.65 mg. per kilogram. No strict comparison between these requirements can be made, however, because of the small number of animals. Furthermore, although racemic α-tocopherol has been reported to function probably equally as well as the natural product (Karrer, Fritzsche, Ringier and Salomon, '38), still it is well to point out that the tocopheryl acetate we used was the racemic product. Nevertheless, it is apparent that intramuscular injection of the water-soluble disodium α-tocopheryl phosphate is at least as effective as oral administration of the dl-α-tocopherol acetate, in striking contrast to the ineffectiveness of the parenteral administration of the oil soluble acetate ester. This suggests that the difference in behavior of orally and parenterally administered

[7] Kindly supplied by Hoffman-LaRoche, Inc.

TABLE 2

Effect of cod liver oil and tocopheryl esters on the progress of muscle dystrophy.

RABBIT NO.	COD LIVER OIL SUPPLEMENT BEGUN ON . . DAY	ACUTE DYSTROPHY IN . . DAYS AFTER C.L.O. TREATMENT	TOCOPHERYL PHOSPHATE INJECTED (TOTAL)	TOCOPHERYL ACETATE FED	PROTECTED FOR . . DAYS	MINIMUM REQUIREMENT	CREATINE RATIO $\left(\frac{\text{CREATINE} \times 100}{\text{TOTAL CREATININE}}\right)$	COMMENTS
	days	*days*	*mg.*	*mg.*	*days*	*mg./kg./ days*		
3	35	6	15				78 at start of treatment; 74 at time of death	Died in 8 days
4	35	9	10		23	0.23	46 at start of treatment; in 3 days down to 10	
8	35	23	12.5		22	0.30	44 at start of treatment; in 3 days dropped to 25, and in 6 days to 9	
11	35	7	5				72, 77 on second day	Died in 2 days
12	35	9	10		22	0.29	58 at start of treatment; down to 8 in 4 days	
15	35	22	15				59 at start of treatment; 74 at time of death	Died on 3rd day
16	35	21	17.5				48 at start of treatment; 12 after 4 days	
19a	35	10	10		16	0.42	54 at start of treatment; 5 after 5 days	
20	35	7	10		29	0.20	41 at start of treatment; 62 next day, and 10 after 2 days	
13	58	6	15		14	0.64	52 at start of treatment; dropped steadily to 9 in 8 days	
17	58	9	10		24	0.20	22 at start of treatment; 37 next day, and 2 on fourth day	
18	58	3	15				68 at start of treatment; 73 at time of death	10 mg. injected first day; 5 mg. second day. Died on third day
14	58	3		10	13	0.65	51 at start of treatment; 7 after 2 days	Fed at one time
19b				10	8	0.77	43 at start of treatment; 1 after 3 days	Fed at one time
1							Creatine ratio wavered between 15 and 25 but returned to normal, about 9	Dystrophy symptoms appeared on 22nd day
2							Creatine ratio high, but still within normal range, for about 2 days; about 24 on 48th and 49th day	Dystrophy symptoms appeared on 28th day
5							Between 64th and 70th day increased up to 40 but dropped back again	Dystrophy symptoms appeared on 19th day
6							Normal whenever tested	Dystrophy symptoms appeared on 22nd day
9							33 on 63rd day but dropped again	Dystrophy symptoms appeared on 20th day
10							Normal whenever tested	Dystrophy symptoms appeared on 26th day
21							Occasional high values (up to 47) with return to normal	Symptoms appeared on 22nd day
22							Normal whenever tested	Symptoms appeared on 17th day. Killed on 61st day because of accidental injury

tocopheryl acetate may possibly be due to a difference in solubility and rate of absorption rather than to a lack of an enzyme necessary to hydrolyze the injected ester.

DISCUSSION

The oil soluble acetate ester of α-tocopherol fed by mouth and the water soluble phosphate ester injected intramuscularly are both effective in curing rabbits which have become severely dystrophic upon the inclusion of cod liver oil in their diet. Mackenzie, Mackenzie and McCollum ('41) using free α-tocopherol under similar experimental conditions found that this had to be fed in very large amounts to produce cures. This may be attributed to the development of rancidity in the intestine when cod liver oil is present in the diet, causing the oxidation and destruction of the free α-tocopherol. The tocopherol esters are known to be stable under conditions where the free alcohol is easily destroyed. While there can be no gainsaying that intestinal rancidity is a factor of considerable importance in the destruction of α-tocopherol, nevertheless the experiments with orally or parenterally administered tocopherol esters do not completely exclude the possibility of an alternative interpretation, that the cod liver oil itself may possibly exert an injurious action (see Morgulis, '38, p. 12) which promotes the dystrophic effect of the E-avitaminosis. This can be decided only by further, especially devised experiments.

We suggest that the water soluble disodium α-tocopheryl phosphate would probably prove itself more suitable than the oil soluble α-tocopherol or its acetate ester now used in clinical studies because of the ease of administration and possibly better utilization. It is quite likely that vitamin E deficiencies develop in some cases not from lack of the substance in the diet but rather from slow and imperfect absorption from the intestines, or even from excessive destruction in the organism. Such conditions could be more effectively remedied by the parenteral administration of an easily absorbable com-

pound, like the α-tocopheryl phosphate. The discordant results from the clinical use of tocopherol in the treatment of certain diseases might be explained, in part at least, on the basis of its variable absorbability from the intestine.

SUMMARY

1. The parenterally administered disodium phosphate ester of α-tocopherol is effective in treating nutritional muscular dystrophy of rabbits in contrast to the ineffectiveness of injected α-tocopheryl acetate.

2. The finding that cod liver oil is necessary in the diet to produce active dystrophy in rabbits has been confirmed and its significance discussed.

3. A simplified and accurate test of creatinuria for detecting active dystrophy has been described.

4. The use of aqueous solutions of the sodium salts of α-tocopheryl phosphate for parenteral administration in clinical treatment of suspected avitaminosis-E has been suggested.

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THE DAILY INTAKE OF ASCORBIC ACID REQUIRED TO MAINTAIN ADEQUATE AND OPTIMAL LEVELS OF THIS VITAMIN IN BLOOD PLASMA [1]

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Neuweiler ('39) estimated that 0.8 mg. ascorbic acid per 100 ml. plasma denoted a good normal value without saturation of the tissues, and this was in agreement with the view of Greenberg, Rinehart and Phatak ('36) that plasma levels of 0.7 to 0.9 mg. ascorbic acid were adequate but not optimal. Farmer and Abt ('38) set 0.7 mg. per 100 ml. as the prescurvy level. In view of these reports the Northwest Nutrition Cooperative Research Group took 0.8 mg. ascorbic acid per 100 ml. as the criterion for judging an adequate state of nutrition as regards ascorbic acid. Their results on the nutritional status of college students with respect to ascorbic acid will be published soon.

In this investigation there have been determined the intakes of ascorbic acid necessary to maintain plasma levels of 0.8 mg. ascorbic acid per 100 ml., as well as saturation levels.

[1] This investigation is part of the regional project of the Northwest States on the ascorbic acid metabolism of college students.

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INTAKES OF ASCORBIC ACID NECESSARY TO MAINTAIN PLASMA LEVELS OF 0.8 MG. PER 100 ML.

Methods

Four college students, two men and two women, 21 or 22 years of age, served as subjects. They were placed on a constant diet practically the same as that used by Belser, Hauck and Storvick ('39) and Todhunter and Robbins ('40). This diet contained weighed amounts of canned beets, carrots, dried prunes, evaporated milk, cheese, and beef; eggs, cereals, butter, nuts and sugar were used ad libitum. Each food was analyzed for ascorbic acid by either the indophenol titration method of Bessey ('38 b), or his photoelectric colorimetric modification of it ('38 a). The prunes and beets were analyzed by the method of McHenry and Graham ('35). This diet provided 8 to 11 mg. ascorbic acid per day. Daily supplements of ascorbic acid [2] were given each morning, directly after a blood sample had been drawn. Plasma ascorbic acid determinations were made daily according to the micro method of Farmer and Abt ('38).

For 3 pre-experimental days a supplement of either a pint of orange juice, or 100 mg. crystalline ascorbic acid was given, in order to be sure that the blood ascorbic acid concentration was above 0.8 mg. per 100 ml. plasma. This guarded against the possibility of the daily ascorbic acid supplement going to make up a previous deficiency. The preliminary period was followed by a 7-day experimental period when the two women received 50 mg. and the two men 60 mg. ascorbic acid per day in addition to the amount contained in the diet. After another 2- or 3-day period on 100 mg. per day to raise the blood level above 0.8 mg. ascorbic acid per 100 ml. plasma, there followed another 7-day experimental period on different supplements of ascorbic acid; and for the women, a third period was carried out some months later.

Results

The blood plasma ascorbic acid values are given in table 1. After each subject had received a constant amount of ascorbic

[2] The ascorbic acid was generously supplied by Merck and Company.

acid for 3 days, the plasma ascorbic acid concentration dropped to approximately a stationary point, providing the plasma level was maintained at approximately 0.8 mg. per 100 ml. or above. The plasma values for the last 3 days on each level of intake are therefore expressed as ranges, rather than being

TABLE 1

Daily blood plasma ascorbic acid levels on different intakes of ascorbic acid.

DAY	SUBJECT MLS — ♀ HEIGHT 154 CM., WEIGHT 45 KG.		SUBJECT LS — ♀ HEIGHT 164 CM., WEIGHT 60 KG.		SUBJECT WL — ♂ HEIGHT 168 CM., WEIGHT 60 KG.		SUBJECT RS — ♂ HEIGHT 176 CM., WEIGHT 75 KG.	
	Supplement to diet	Blood plasma	Supplement to diet	Blood plasma	Supplement to diet	Blood plasma	Supplement to diet	Blood plasma
	Series I							
	mg.	*mg./100 ml.*	*mg.*	*mg./100 ml.*	*mg.*	*mg./100 ml.*	*mg.*	*mg./100 ml.*
1–3	100	0.92–1.06	100	1.14–1.19	100	1.08–1.16	100	0.92–1.05
4	50	1.02	50	1.12	60	1.19	60	1.06
5	50	1.00	50	1.10	60	1.04	60	1.02
6	50	0.89	50	0.95	60	1.03	60	0.85
7	50	0.82	50	0.89	60	1.01	60	0.96
8–10	50	0.82–0.85	50	0.92–0.99	60	0.80–0.82	60	0.87–0.74
11	100	0.82	100	0.86	100	0.84	100	0.66
12	100	1.00	100	1.00	100	1.02	100	0.80
13	40	1.02	40	1.06	50	1.00	170	0.86
14	40	0.99	40	1.03	50	0.95	80	0.87
15	40	0.95	40	0.91	50	0.83	80	0.79
16	40	0.93	40	0.89	50	0.83	80	0.85
17	40	0.82	40	0.86	50	0.82	80	0.80
18–20	40	0.82–0.86	40	0.86–0.90	50	0.76–0.77	80	0.72–0.81
	Series II							
1–2	100	0.94–1.05	100	0.98–1.06				
3	30	0.84	30	0.85				
4	30	0.63	30	0.84				
5	30	0.54	30	0.78				
7–9	30	0.74–0.59	30	0.90–0.64				

given as individual values for these days. The women, when on the 30 mg. supplement, and the man, RS, when receiving 60 mg. ascorbic acid as the supplement, did not show this leveling off. The ascorbic acid concentration of the plasma continued to go down in those cases, apparently showing too

low an intake. The daily intakes (food sources plus supplements) necessary to maintain the level of 0.8 mg. ascorbic acid per 100 ml. were therefore considered to be about 49 mg. ascorbic acid for MLS, 38 mg. for LS, between 59 and 69 mg. for WL and at least 89 mg. for RS. These intakes corresponded approximately to 1.1, 0.8, 1.1 and 1.2 mg. per kilogram, respectively.

One other subject, ER, in a different series reported below, on an intake of 61 mg. ascorbic acid showed a plasma concentration of 0.81 mg. per 100 ml. This amounted to an intake of 1.0 mg. per kilogram. The average requirement with its standard deviation to maintain this blood level was therefore 1.0 ± 0.13.

INTAKES OF ASCORBIC ACID NEEDED FOR "TISSUE SATURATION"

Methods

A similar investigation was begun on four women subjects, to determine the intake of ascorbic acid necessary to maintain tissue saturation evidenced by plasma ascorbic acid concentration and excretion of ascorbic acid in response to a test dose. Unfortunately, one of the student subjects left school before the investigation was completed, but work was continued on the remaining three persons.

The method as described by Belser, Hauck and Storvick ('39) was followed. Each subject was brought to a state of tissue saturation by the daily ingestion of 200 mg. ascorbic acid for 3 or more days. A test dose of 400 mg. was then given and the excretion of ascorbic acid during the next 24 hours measured. The individual was then placed on a constant intake of ascorbic acid, e.g., 60 mg. for 6 days, after which the test dose of 400 mg. was again administered and excretion during the next 24 hours measured. After resaturation again, the test period was repeated with a different supplement, and the test dose once more administered. Blood plasma ascorbic acid determinations were made by the micro method of Farmer and Abt ('38) before each test dose and 24 hours after. With

two of the subjects, daily blood determinations were made through at least two of the experimental periods.

The basal diet, which was the same as that reported above but with different lots of canned goods, provided a total of 11 mg. ascorbic acid per day.

Urine was collected in quart jars in which the following preservative as recommended by Sendroy (private communication) had been placed: 75 ml. 5N sulfuric acid, 2 ml. toluene and 2 ml. 8-hydroxyquinoline (1.45% alcoholic solution). Urine preserved in this way retained 95% of its ascorbic acid. The ascorbic acid concentration in the urine was determined by titration with a solution of 2,6-dichlorophenol indophenol, standardized daily, as well as by Bessey's method ('38 a) using the Evelyn photoelectric colorimeter. There was close agreement between the values obtained by the two methods.

Results

The lowest excretion in the 24 hours after the test dose of 400 mg. ascorbic acid when the subject was known to be in saturation (i.e., after receiving 200 mg. for at least 3 days) was taken as index of saturation for that individual. Likewise the lowest blood plasma ascorbic acid level when the subject was in known saturation was considered as the saturation level for that subject. The values (table 2) for excretion and plasma level per 100 ml., respectively, were as follows for each subject: MF, 272 and 1.0 mg.; AG, 267 and 1.2 mg.; ER, 291 and 1.1 mg.; and LS, 325 and 1.1 mg.

In no case except for subject AG, did the excretion of ascorbic acid in response to the test dose parallel the previous intake of ascorbic acid. For MF and ER, at no time after an experimental period did the excretion after the test dose reach the lowest level of excretion after saturation. After AG had ingested 111 mg. a day for 6 days, the excretion after the test dose amounted to 251 mg., an amount very near to the 267 mg. excreted when she was in known saturation.

In general, the blood plasma levels were higher with the higher intake and paralleled the intake more closely than did response to the test dose. When plasma levels were determined daily, they were quite consistent at any one level of intake. For instance, when MF was ingesting 111 mg. ascorbic acid per day, her plasma values on successive days were 0.87, 0.87, 0.84, 0.88, 0.89 and 0.94 mg. per 100 ml., and when her

TABLE 2

Blood plasma ascorbic acid levels after a constant intake of ascorbic acid for 3 to 6 days followed immediately by a test dost of 400 mg.; and plasma levels and excretion after the test dose.

Daily intake	No. days at that level	BLOOD PLASMA LEVELS Before test dose	After test dose	Excretion in 24 hours after test dose
mg.		*mg./100 ml.*		*mg.*
Subject MF — Height 163 cm.; Weight 61 kg.				
200	3	0.99	1.01	393
200	3	1.10	1.10	318
200	3	1.22	1.06	334
200	3	1.06	0.99	272
61	6	0.63	0.88	237
91	6	0.91	0.97	190
111	6	0.90 [1]	0.94	225
131	6	1.05 [1]	1.06	223
Subject AG — Height 165 cm.; Weight 66 kg.				
200	3	1.26	1.23	328
200	3	1.31	1.35	267
91	6	1.03 [1]	1.14	212
111	6	1.18	1.22	251
Subject ER — Height 165 cm.; Weight 59 kg.				
200	3	1.29	1.22	393
200	3	1.12	...	381
200	3	...	1.19	291
200	3	1.18	1.14	299
61	6	0.81	1.05	236
81	6	0.93	0.99	216
111	6	1.12	1.20	255
Subject LS — Height 152 cm.; Weight 52 kg.				
200	3	1.20	1.19	430
200	3	1.18	1.10	325
61	6	0.89	0.92	279
81	6	0.81	0.88	218

[1] Average of last 3 days.

intake was 131 mg. the daily plasma values amounted to 1.09, 0.92, 1.17, 0.98, 1.00 and 1.06 mg. per 100 ml. It is therefore our opinion that the plasma values represent a better criterion than those for excretion for judging saturation after a test dose.

The daily intakes of ascorbic acid necessary to maintain tissue saturation amounted to 131 mg., 111 mg., and 111 mg.,

respectively, as measured by blood plasma ascorbic acid concentration; these represented intakes of 2.0, 1.8 and 1.7 mg. per kilogram of body weight for the three subjects.

These intakes of ascorbic acid necessary to maintain saturation are comparable with those reported by other workers as follows: Belser, Hauck and Storvick ('39), 70 to 100 mg.; Todhunter and Robbins ('40), 90 to 110 mg.; and Ralli, Friedman and Sherry ('39), 100 mg.; and with the 100 mg. or more estimated by Smith ('38) to constitute a luxus consumption level. These values, ranging from 1.0 to 1.8 mg. per kilogram, with those reported above, 1.7 to 2.0 mg. per kilogram, indicate that there is no relation between body weight and ascorbic acid required to maintain saturation levels in the blood.

SUMMARY AND CONCLUSIONS

The daily intake of ascorbic acid necessary to maintain a blood level of 0.8 mg. ascorbic acid per 100 ml. plasma has been determined for five subjects. This amount was 38–49 mg., 49 mg. and 61 mg., respectively for three women; and 69 mg. and 89 mg. per day for two men, corresponding to intakes of 0.8 to 1.2 mg. per kilogram, averaging 1.0 ± 0.14.

In three different subjects, there were measured the intakes of ascorbic acid necessary to maintain tissue saturation as determined by blood plasma levels. These values amounted to 111, 111 and 131 mg., respectively, or 1.7, 1.8 and 2.0 mg. per kilogram body weight.

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STUDIES ON THE VITAMIN B COMPLEX IN THE NUTRITION OF THE DOG [1]

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TWO FIGURES

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Studies on the vitamin B complex in the nutrition of the dog have been carried on in this laboratory using a purified casein — sucrose ration supplemented with synthetic vitamins and liver extracts. Deficiencies of riboflavin (Axelrod, Lipton and Elvehjem, '40, '41), nicotinic acid (unpublished data), pyridoxine (McKibbin, Madden, Black and Elvehjem, '39; McKibbin, Schaefer, Frost and Elvehjem, '42), pantothenic acid (McKibbin, Black and Elvehjem, '40), and choline (Schaefer, McKibbin and Elvehjem, '41) have been studied or demonstrated. During our earlier studies on pantothenic acid deficiency evidence was obtained for the existence of two additional factors essential for the dog — one stable to alkali, the other labile to alkali. Recently we have shown that this work was probably complicated with choline deficiency (Schaefer, McKibbin and Elvehjem, '41) and that choline was undoubtedly one of these factors. The availability of synthetic pantothenic acid has enabled us to continue the study of the unknown factor or factors.

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EXPERIMENTAL

The basal ration and general experimental procedure have been described previously (Schaefer, McKibbin and Elvehjem, '41). The ration had the following percentage composition: sucrose 66, acid-washed casein 19, cottonseed oil 8, cod liver oil 3, and salt mixture 4. This ration was supplemented with 100 μg. per kilogram of body weight per day of thiamine chloride and riboflavin, 2 mg. of nicotinic acid, 60 μg. of pyridoxine hydrochloride, and 500 μg. of calcium pantothenate. Weanling puppies were used in all of the experiments unless otherwise specified.

During our earlier studies on pantothenic acid deficient rations we observed that animals receiving liver extract which had been treated with ether would not respond consistently to purified concentrates of pantothenic acid (McKibbin, Black and Elvehjem, '40). This suggested that the acid ether extraction procedure removed some other essential factor. It was thought advisable, therefore, to use the acid ether extract or the acid ether residue in basal rations containing synthetic pantothenic acid with the idea of producing an uncomplicated deficiency of either one of the two unknown factors.

Accordingly, four pups were given the basal ration supplemented with 2% of the acid ether residue of liver extract "fraction D."[2] One of these animals received in addition 50 mg. of choline chloride per kilogram of body weight per day. Another pup received 2% of the acid ether extract of fraction D supplemented with choline as above. Of these five dogs, four showed excellent growth and appeared in normal condition over the assay periods of 167, 131, 79 and 77 days. The fifth dog, receiving the acid ether residue unsupplemented with choline, showed complete growth failure by the thirty-ninth day. Growth was resumed when a lead acetate filtrate of liver extract powder 1:20[3] was added to the ration.

[2, 3] The Wilson Laboratories.

From these results it is evident that, although use of these liver fractions can result in a demonstrable deficiency, these fractions are not dependable for assay of liver extracts containing these factors. It is probable that much of the choline in liver extracts is not removed with acid ether since two of the dogs receiving the acid ether residue grew normally without added choline, whereas we have found that on a choline-free ration deficiency can be expected in 3 to 6 weeks.

In our earlier studies we also observed that liver extracts treated with alkali to remove pantothenic acid would not permit growth indefinitely with the periodic addition of pantothenic acid concentrates (McKibbin, Black and Elvehjem, '40). These results suggested that there was an essential factor in liver extract which was labile to alkali. It was therefore decided to study this factor using alkali-treated liver extracts.

Three littermate puppies were used in this experiment. They received the basal ration supplemented with 50 mg. of choline chloride per kilogram of body weight per day. In addition one received 2% of the alkali treated fraction D, another received 2% of the acid acetone extract of fraction D (McKibbin, Madden, Black and Elvehjem, '39), and the last received 2% of this fraction treated with alkali. The alkali treatment of these fractions was carried out by diluting ten times with water, adjusting to neutral with litmus, adding solid KOH to a concentration of 1 N and then heating on the steam bath for 1½ hours at 88–95°C. The preparations were then neutralized with acid, the salts precipitated with alcohol, and the filtrates concentrated under reduced pressure. These dogs all showed excellent growth and appeared normal throughout the assay periods of 115, 204 and 115 days. The comparison of these results with those of our earlier studies indicates that the alkali labile factor encountered was undoubtedly choline.

A choline deficiency (Schaefer, McKibbin and Elvehjem, '41) was then produced on the synthetic ration supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, and

pantothenic acid. The puppies used in the choline experiment were then continued on the basal ration supplemented with 50 mg. of choline chloride per kilogram of body weight per day. Dogs 193, 195, 196 and 197 were given this ration while dog 192 was given in addition 2% of liver extract fraction D. The growth of the first four dogs was suboptimal and erratic while that of dog 192 was fairly consistent. After the dogs had received this ration 4½ months without developing an acute

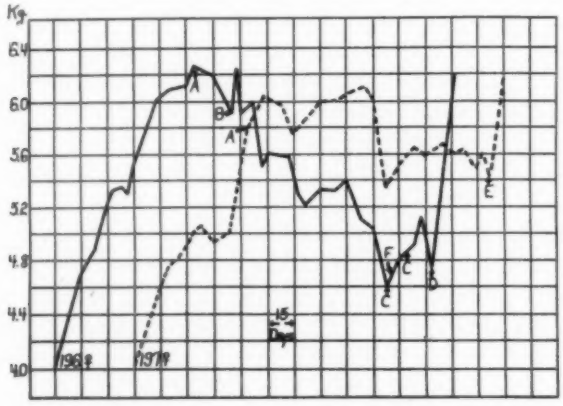

Fig. 1 Response of two growing dogs to the replacement of the acid-washed casein with the alcohol-extracted casein in the basal synthetic ration. A. Changed to purified casein ration; B. Started 50 mg. per kilogram body weight per day of i-inositol; C. Oral administration of i-inositol and p-amino benzoic acid; D. Started 3% liver extract powder 1:20 in ration; E. Started 6–10% whole liver powder in ration; F. Started intravenous injections of glutamine.

deficiency, it was decided to purify the casein further. This was done by refluxing with 95% ethanol for four 12-hour periods. The response of dogs 196 and 197 to this change is shown in figure 1. After 3 weeks there was a slow steady decline in weight until at the end of 3½ months the dogs had lost approximately 25% and 11% of their body weight, respectively. In contrast to this, dogs 193 and 195 maintained on the acid-washed casein ration continued to grow to weights of 6.8 and 7.3 kg. Inositol feeding at 50 mg. per kilogram

per day over a 16-day period failed to prevent this loss in weight of dog 196. Later two oral administrations, each of 200 mg. p-amino benzoic acid and 1 gm. of inositol, along with three intravenous injections of 50-mg. of glutamine in an 11-day period produced only a slight increase in weight. The addition of 3% liver extract powder 1:20 to the basal ration produced a complete and immediate restoration of the lost weight. The addition of 6% and then 10% of whole dry liver to the ration of dog 197 restored the original weight of this dog. Another dog (no. 198) given this purified casein ration for 1½ months also reached a growth plateau. After 3 months more this ration was supplemented with 3% liver extract powder 1:20 which resulted in a weight increase of 0.8 kg. (13% of body weight) in 12 days.

The apparent nutritive differences between the alcohol extracted casein and that used in our previous studies suggested an experiment using this purified casein from the start. Accordingly, a litter of five dogs was placed on this ration supplemented with the six synthetic vitamins at levels used in the previous experiments. One dog (no. 202) received in addition 2% liver extract powder 1:20. The response of these dogs is shown in figure 2. Choline was withheld from dog 207 until deficiency appeared, then it was added to the ration and growth was resumed. It can be seen that only one of the dogs not receiving liver extract grew at a rate comparable to that of the dog receiving liver extract. The others reached a growth plateau 2 to 5 kg. below this weight.

The results with five dogs from various litters placed on the acid-washed casein ration were different from the above results using alcohol-extracted casein. Dogs nos. 186, 188, 190, 191, and 174 received liver supplements for periods of 3, 3, 4½, 5, and 5½ months, respectively. After these periods all liver supplements were discontinued and the ration supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline. These animals have been maintained on this ration in normal condition for 9½, 9½, 4½, 5, and 5½ months, respectively. Of these dogs,

nos. 186, 188, 190, and 191 were subjected throughout these periods to the additional strain of weekly phlebotomy in our hemorrhagic anemia studies and they are still in excellent condition.

In some growing dogs, however, the acid-washed casein ration supplemented with synthetic vitamins frequently shows failure to support growth. Dog 172 receiving all six vitamins

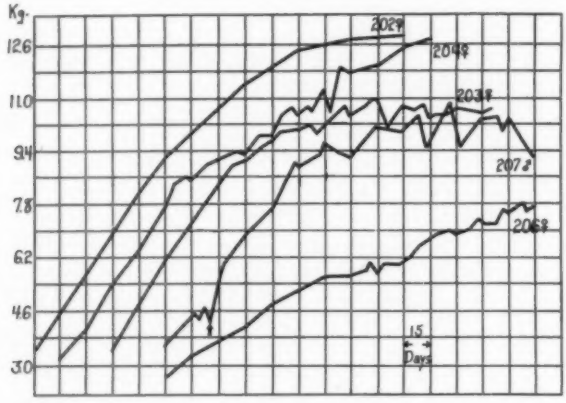

Fig. 2 Growth of five littermate puppies on the purified ration containing alcohol-extracted casein. Dogs 204, 203, 207 and 206 received basal ration with synthetic vitamins only; dog 202 received in addition, 2% liver extract powder 1:20. ↑ = started choline 50 mg. per kilogram body weight per day.

developed a growth plateau which was corrected by the addition of liver extract to the ration as shown in table 1. Dogs 179 and 200 received all the synthetic vitamins but nicotinic acid. Anorexia with concomitant loss of weight developed which could not be corrected with nicotinic acid but was immediately corrected by supplementing the ration with liver extract as shown in table 1.

DISCUSSION

From these experiments it can be seen that no rapidly fatal deficiency appears on the purified ration supplemented with the six synthetic B vitamins, whereas in deficiency of any one

of these six, symptoms appear in young puppies in 3 to 6 weeks, in older dogs in 1 to 3 months. Deficiency of additional factors may not appear at all on the basal ration containing our acid-washed casein, whereas this ration always produces deficiency of any one of the above-mentioned six synthetic

TABLE 1

Behavior of three growing dogs receiving the basal acid-washed casein ration and various supplements.

DOG NO.	LITTER	RATION AND SUPPLEMENTS	INITIAL WEIGHT	TEST PERIOD	TOTAL GROWTH	REMARKS
			kg.	*days*	*kg.*	
172	G	Basal acid-washed casein ration	4.5	152	3.0	Good growth followed by a plateau for 57 days
172		3% liver extract powder 1: 20 (The Wilson Laboratories)	7.5	26	1.15	Excellent growth
179	H	Basal acid-washed casein; received no nicotinic acid	4.2	30	—0.5	Decline in weight
179		18.4 mg. nicotinic acid	3.7	4	+0.5	Rapid growth
179		Basal	4.2	26	—0.4	Decline in weight
179		20 mg. nicotinic acid	3.8	15	—0.15	Decline in weight
179		15.4 mg. nicotinic acid	3.65	4	0	No response
179		10 gm. 1: 20 liver extract powder	3.65	16	+0.75	Excellent growth
200	I	Basal acid-washed casein ration; received no nicotinic acid	1.7	12	+0.05	Poor growth
200		10 mg. nicotinic acid	1.75	11	0.10	No response
200		30 mg. nicotinic acid	1.85	9	—0.05	No response
200		2% liver fraction "D" (The Wilson Laboratories)	1.75	40	2.15	Good growth

vitamins. However, if the acid-washed casein is purified further by alcohol extraction, deficiency characterized by anorexia and loss of weight usually appears after 1 to 3 months. In several dogs we have observed a slight to marked greying of the hair. From our studies on pantothenic acid

deficiency in dogs (unpublished data) we feel fairly certain that the possibility of this greying being due to pantothenic acid deficiency may be ruled out.

There seems to be, therefore, a qualitative difference between the apparent requirement of the dog for thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline, and that of the other factors in the vitamin B complex when one compares the speed of onset and the acuteness of the deficiency of the first six members with that of the other factor in liver extract. The dietary requirement for the liver factor may be lessened by the ability of the animal to effect a partial synthesis in the tissues or in the intestine by bacteria.

If intestinal synthesis of this factor accounts for the differences observed in apparent requirement between this factor and the other members of the vitamin B complex, any dietary changes which might affect the intestinal flora might be expected to interfere with the synthetic processes. The results obtained with dogs 179 and 200 receiving no nicotinic acid might be explained on the basis that dietary nicotinic acid was essential in some manner for the bacteria responsible for the synthesis of the liver factor. When nicotinic acid was removed from the diet synthesis of the liver factor was curtailed and the dogs would no longer respond to nicotinic acid but would respond to liver extract. The addition of certain bacteriostatic drugs such as sulfaguanidine to the ration might also interfere with these syntheses as suggested by experiments with rats (Black, McKibbin and Elvehjem, '41). Such an approach might throw considerable light on this question.

The identity of the substance or substances in liver extract responsible for the improvements recounted above cannot be established from these studies. Inositol has been shown to be essential in the nutrition of yeast (Koser and Saunders, '38) and mice (Woolley, '40). The role of inositol in rat and chick nutrition is less clear. From our results it seems apparent that inositol alone at levels comparable to those found effective in other species does not replace liver extract.

The suggested role of p-amino benzoic acid in the nutrition of the rat and chick (Ansbacher, '41) as well as its capacity to neutralize the bacteriostatic action of sulfanilamide on certain microorganisms suggests the possibility of its importance in dog nutrition. The use of glutamine was suggested by its growth-stimulating action on certain bacteria (Feeney and Strong, in press). Preliminary evidence indicates that these substances will not replace liver extract when given in combination with inositol. All these substances, however, may deserve further consideration as essential elements in dog nutrition and will be checked further.

The possible role of biotin in the nutrition of the dog is still obscure. We have not as yet substituted biotin concentrates for liver extract in these studies.

Studies on pantothenic acid and nicotinic acid deficiencies on this acid-washed casein ration supplemented only with synthetic vitamins are now in progress.

SUMMARY

1. Dogs receiving a highly purified vitamin B complex free ration supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline may grow erratically and suboptimally. Growth may be increased by the addition of liver extract to the ration.

2. By further purification of the casein in this ration growth is usually arrested and anorexia and loss of weight occur. This condition is curable by liver extract. Preliminary evidence indicates that the condition is not curable by mixtures of inositol, p-amino benzoic acid, and glutamine.

3. The onset of this deficiency is much slower and more chronic than is observed with those six B vitamins mentioned above.

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AVAILABILITY TO RATS OF PHOSPHORUS IN RED CLOVER HAYS OF WIDELY VARYING PHOSPHORUS CONTENT

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THE PROBLEM

Previous work with rats (Williams, MacLeod and Morrell, '40 a, b) indicated that animals fed a diet containing an amount of phosphorus minimal for growth, one-half of which came from a low phosphorus hay, gained less weight and stored less phosphorus over a 30-day experimental period than animals fed a diet containing the same amount of phosphorus, one-half of which came from a high phosphorus hay of the same type. Similar results were obtained whether the hays used were lespedeza sericea, alfalfa, soybean hay, or red clover. This work has now been extended to show the effect on white rats of the addition of (1) a liberal phosphorus supplement to each ration containing the hays of differing phosphorus content, and (2) the ash of the two hays to a stock ration in amounts equivalent to the hay in the preceding rations. The first procedure was adopted in an attempt to determine whether or not the mere addition of a phosphorus supplement to the diet containing the low phosphorus hay would make it as adequate as that containing the high phosphorus hay. The purpose of adding the phosphorus of the hay in the form of its ash rather than as the whole product was to eliminate from consideration all differences in bulk and in organic materials. Previous work has noted the marked

differences in amounts of feces due to the different diets, and has recognized the possibility of differences in quality not brought out by routine analysis of the two hays, such as in the fraction designated "nitrogen-free extract." If differences in animal response still persisted when the hay was fed as ash, the explanation, in part at least, of the cause for the differences would have been narrowed down to the mineral fraction of the hays.

EXPERIMENTAL PROCEDURE

Essentially the same experimental procedure was used for these experiments as that reported in earlier articles. The red clover hays were the same as used previously, the phosphorus analyses being as follows: the low phosphorus red clover, 0.12% (0.27% P_2O_5); and the high phosphorus red clover, 0.31% (0.71% P_2O_5). Tables 1 and 2 show the composition of the experimental diets. In the diets containing the red clover hay, all essential nutrients were present in adequate amounts, including phosphorus which was at the level of

TABLE 1

Percentage composition of experimental rat diets.

COMPONENT	RED CLOVER HAY			RED CLOVER HAY ASH[1]		
	Control	Low P hay	High P hay	Control	Low P ash	High P ash
	%	%	%	%	%	%
Hay or ash		69	26		4.7	1.7
Lactalbumin [2]	18	11	15	18	18	18
Starch [2]	49	6	34	49	44.3	47.3
Agar	19	0	11	19	19	19
Salt mixture [3]	4	4	4	4	4	4
Butterfat	8	8	8	8	8	8
Cod liver oil	2	2	2	2	2	2

[1] Hay ash diets for rats in series I and II were identical except that agar was not included in the diets for series II and the starch was correspondingly increased.

[2] The same amount of yeast extract was included in comparable diets, being incorporated with the starch or lactalbumin.

[3] Osborne and Mendel salt mixture modified to give desired amounts of phosphorus and calcium, and by the addition of copper sulphate.

0.32% of the whole diet. In contrast to the diets in the previously reported studies which contained only a minimal amount of phosphorus at the level of 0.16%, this represented a liberal phosphorus supplement for each diet. In the diets containing the red clover ash, all essential nutrients were present in adequate amounts except for phosphorus which was at the

TABLE 2

Distribution of foodstuffs among the various constituents of the red clover hay and the red clover hay ash diets.

DIETARY FACTOR	RED CLOVER HAY DIETS			RED CLOVER HAY ASH DIETS [1]		
	Control	Low P hay	High P hay	Control	Low P ash	High P ash
	%	%	%	%	%	%
Protein:						
Hay	0	7	3	0	0	0
Lactalbumin	18	11	15	18	18	18
Total	18	18	18	18	18	18
"N-free extract":						
Hay	0	28	10	0	0	0
Starch	49	6	34	49	44.3	47.3
Total	49	34	44	49	44.3	47.3
Fat:						
Butterfat	8	8	8	8	8	8
Cod liver oil	2	2	2	2	2	2
Fiber:						
Hay	0	19	8	0	0	0
Agar	19	0	11	19	19	19
Total	19	19	19	19	19	19
Calcium:						
Hay	0	0.959	0.263	0	0.967	0.260
Lactalbumin	0.036	0.022	0.030	0.036	0.036	0.036
$CaCO_3$	0.887	0	0.718	0.868	0	0.686
Total	0.923	0.981	1.011	0.904	1.003	0.982
Phosphorus:						
Hay	0	0.080	0.080	0	0.079	0.076
Lactalbumin	0.027	0.017	0.023	0.027	0.027	0.027
H_3PO_4	0.280	0.218	0.206	0.132	0.066	0.058
Total	0.307	0.315	0.309	0.159	0.172	0.161
Ca/P ratios	3.0	3.1	3.3	5.7	5.8	6.1

[1] See footnote 1, table 1.

minimal level of 0.16%, one-half of the total coming from hay ash and the other half from a salt mixture. The hay was ashed [1] at first over an open flame and finished in a muffle furnace at 600°C. Two series of animals were run on the ash diets. Series I had agar in all diets in an amount to equal the fiber content of the corresponding diets in which the hay was fed. Series II had no agar in the diets and the starch content was correspondingly increased. The Ca/P ratios for the diets containing the phosphorus supplement were approximately 3:1 and for the diets containing the hay ash, they were approximately 6:1 which is the same ratio as published previously for the comparable diets in which red clover hay itself was fed rather than the ash.

The triplicate feeding method with equalized food consumptions was employed as in previously reported work. The animals were 30–31 days old when started on the experimental diets on which they were continued for 30 days. Rate of growth and phosphorus retention were again used as criteria in judging results.

RESULTS

Table 3 summarizes the average results obtained for all animals. It also includes data published earlier for rats on a similar ration using the same red clover hays in which the total phosphorus was at the level of 0.16%. It will be noted that for both males and females, the rats which received phosphorus at the 0.32% level, one-fourth of which came from a low phosphorus hay, were conspicuously less thrifty animals than either the controls or those deriving one-fourth of the phosphorus from a hay high in phosphorus content whether judged on the basis of gain in weight, body length, or the amount of phosphorus retained in the body. A statistical analysis by "Student's" method as given by Goulden ('39) reveals significant differences between the animals on the low phosphorus and high phosphorus hay diets both as regards

[1] The hay was ashed by Mr. B. W. Hatcher, Asst. Soil Chemist, Agricultural Experiment Station, University of Tennessee.

TABLE 3

Summary of average results for experimental rats in growth and phosphorus intake and retention.

EXPERIMENTAL GROUP	NO. OF CASES	WEIGHT			LENGTH OF TRUNK	TOTAL FECES DRY WEIGHT	FOOD CONSUMPTION	TOTAL P IN FOOD	P IN RAT AT 60–61 DAYS	P IN RAT AT 30–31 DAYS (ESTIMATED)	P STORED FROM FOOD	P FROM FOOD
		Initial net	Final net	Gain net								
		gm.	*gm.*	*gm.*	*cm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*%*
Red clover hay (total P — 0.16%)												
Males												
Control	5	51	105	54	15.4	57	239	0.379	0.535	0.272	0.263	69
Low P	5	51	63	12	13.4	121	239	0.389	0.372	0.271	0.101	26
High P	5	52	92	40	14.6	91	241	0.387	0.492	0.274	0.218	56
Red clover hay (total P — 0.32%)												
Males												
Control	5	56	150	94	16.4	59	274	0.841	0.866	0.295	0.571	68
Low P	5	56	84	29	14.0	145	274	0.861	0.556	0.295	0.262	30
High P	5	56	133	73	15.4	95	277	0.856	0.747	0.295	0.452	53
Females												
Control	5	53	118	65	15.9	52	245	0.752	0.771	0.280	0.491	65
Low P	5	54	72	18	13.7	133	248	0.780	0.521	0.286	0.235	30
High P	5	53	107	54	15.0	86	250	0.772	0.685	0.281	0.404	52
Red clover ash (series I)[1]												
Males												
Control	5	56	79	23	14.4	40	165	0.262	0.492	0.296	0.196	75
Low P	5	56	61	5	13.2	39	157	0.270	0.386	0.296	0.091	34
High P	5	56	78	22	14.3	41	165	0.265	0.463	0.295	0.168	63
Red clover ash (series II)[1]												
Males												
Control	5	58	91	33	15.0	6	181	0.288	0.563	0.305	0.258	90
Low P	5	58	75	17	14.3	9	175	0.304	0.454	0.305	0.149	49
High P	5	58	88	30	15.0	8	180	0.303	0.518	0.306	0.211	70

[1] See footnote 1, table 1.

growth and phosphorus retentions; the probability being less than 1 in 100 that the observed differences are due to chance. If these results are compared with those reported in detail in an earlier paper with the same red clover hay without a phosphorus supplement, i.e., at the 0.16% phosphorus level (table 3), it can be seen that there is an improvement in growth and phosphorus retention of animals on diets containing 0.32% phosphorus over those on corresponding diets containing 0.16% phosphorus. However, the same differences between those receiving part of the phosphorus from the low phosphorus hay and those receiving part from the high phosphorus hay are apparent regardless of the amount of phosphorus in the diet. For male rats on the rations at the 0.16% phosphorus level, the percentages of phosphorus retained from the total phosphorus of the diet for the control, low phosphorus, and high phosphorus hay diets were, respectively, 69, 26, and 56; and for corresponding diets at the 0.32% level they were 68, 30, and 53. It seems possible that the constituents of the low phosphorus hay responsible for the impairment in utilization of phosphorus may act by combining with this element to form insoluble compounds. Elements which are known to form insoluble compounds with phosphorus (Schmidt and Greenberg, '35) and which have been found to be present in the hays used are iron, aluminum, barium, and calcium in excess. Quite a body of literature may be quoted giving evidence that the presence of excessive amounts of iron and aluminum prevents the proper utilization of phosphorus (e.g., Waltner, '27; Cox, Dodds et al., '31; Brock and Diamond, '34; Deobald and Elvehjem, '35; Jones, '38; Rehm and Winters, '40; Freeman and Freeman, '41). A spectrographic analysis [2] of the ash of the two hays indicated the presence of both iron and aluminum. Chemical analyses were subsequently made to determine the relative amounts of these substances in the two hays, using the A.O.A.C. method for plant materials in which the iron and aluminum were weighed

[2] Spectrographic analysis made in Dr. R. V. Allison's laboratory at the University of Florida.

as phosphates. Table 4 presents the experimental data obtained on the basis of these analyses. Assuming for purposes of comparison that all the phosphates were in the form of iron phosphates (calculated as Fe PO_4) and then as aluminum phosphates (calculated as Al PO_4), it was found that the phosphorus contents represented by these percentages of phosphate were from 0.06 to 0.08% for the high phosphorus hay and from 0.09 to 0.11% for the low phosphorus hay. In the high phosphorus hay there was a total phosphorus content of 0.31% and therefore, even though all the iron and aluminum present combined to form insoluble phosphates, there would

TABLE 4

Comparison of the percentages of iron and aluminum phosphates[1] in the low phosphorus and high phosphorus hays.

	0.12% P HAY	0.31% P HAY
Per cent Fe — and $AlPO_4$ in ash	6.23	4.33
Per cent ash in hay	7.18	6.82
Per cent Fe — and $AlPO_4$ in hay	0.447	0.295
Per cent P involved if all PO_4 is $FePO_4$	0.089	0.061
Per cent P involved if all PO_4 is $AlPO_4$	0.110	0.075
Per cent P involved if PO_4 is one-half $FePO_4$ and one-half $AlPO_4$	0.100	0.068
Per cent P left in hay if all possible combined as one-half $FePO_4$ and one-half $AlPO_4$	0.02	0.24

[1] Calculated as $FePO_4$ and $AlPO_4$ respectively.

still be a large margin of safety of phosphorus available to the animal. In the low phosphorus hay, on the contrary, with a total phosphorus content of 0.12%, a complete precipitation of phosphorus by the iron and aluminum would leave little or practically no phosphorus available to the animal. If the relationships between phosphorus content and iron and aluminum content noted in these particular hays prove to have a correlation as regards hays of varying phosphorus content grown on different soil types, we believe that a chemical explanation, or at least partial explanation, may have been found for the differences in the availability of phosphorus previously noted within the same type of forage. It is recognized that other

substances besides iron and aluminum, such as barium, fluorine, or excess calcium, may take part in this depressing effect.

This explanation is in essential agreement with that given by Shields et al. ('40) for differences in utilization of calcium in various food materials. These authors state that "the constituents of vegetables responsible for the impairment in calcium utilization act either by combining with the calcium to form insoluble compounds, as the oxalates of foods are known to do, or to modify unfavorably the reaction of the intestinal contents, or to increase intestinal motility. These factors must account for the so-called 'associative effects' of foods in digestion, whereby one food will impair the digestibility of another."

The results obtained from the rats fed the red clover hay in the form of ash give further corroborative evidence for the above interpretation. In this case the three experimental rations were nearly identical except that in the control ration all the phosphorus was supplied by a salt mixture and in the others one-half the phosphorus was supplied by a small amount of low phosphorus hay ash in one case and by a smaller amount of high phosphorus hay ash in the other. Any interfering minerals present in the ash would have a chance to combine immediately with the phosphorus from any source without elapse of time for the digestion of the organic cellular matter of the hay. This more intimate mixing of the elements in the ash may be the explanation for the drastic effect of the low phosphorus ash diet on the appetite of the rats to which it was fed. They ate barely enough to maintain their weight throughout the experimental period and thus restricted the food consumption of their litter mates in the trio. The small food consumption would seem to account for the small gain in weight for all rats. The differences in growth of the animals on the hay ash in series I and II may be related to the fact that in the first series agar was included in the ration to make the fiber content the same as when the hay itself was fed and in the second series, the agar was omitted. Again we note a

significant difference between rate of growth and phosphorus retention in the animals receiving the low phosphorus hay ash as compared with those receiving the control diet or the diet containing the high phosphorus hay ash. In the first series, the percentages of phosphorus retained from the total dietary phosphorus were comparable to those obtained when one-half the phosphorus was supplied as hay, the values being for the control, low phosphorus ash, and high phosphorus ash diets, respectively, 75, 34, and 63. In the second series the percentages for the corresponding diets were higher, namely, 90, 49, and 70, but in both series there was a marked difference between the rats fed the low phosphorus ash and those fed the control and high phosphorus ash diets. For the two series, the probability was about 1 in 100 that the observed differences were due simply to chance variations. These results seem to indicate that at least a partial explanation for the differences in the availability of phosphorus in these red clover hays of varying phosphorus content may be assigned to the mineral fraction of the hays in question. The fact that the total amounts of feces eliminated on the three diets compared were almost identical seems to rule out this factor as a causative agent.

SUMMARY OF RESULTS

1. When the experimental diets contained all nutrients including phosphorus at the same and optimal levels of adequacy, the phosphorus of a diet containing low phosphorus red clover hay was less available to the rat for growth and bone development than the phosphorus of a diet containing high phosphorus hay of the same type. Evidence is presented indicating that the amounts of iron and aluminum present in the low phosphorus hay are sufficient to be a possible disturbing factor in the utilization of the phosphorus by the formation of insoluble phosphates.

2. When the experimental diets contained phosphorus at a minimal level of adequacy and all other nutrients at adequate levels and essentially the same for all diets, the phosphorus

of a diet containing low phosphorus red clover hay supplied in the form of ash was less available to the rat for growth and bone development than the phosphorus of a ration containing high phosphorus red clover hay ash.

CONCLUSION

The present study gives some evidence for believing that the observed differences between animals receiving the low phosphorus hay and those receiving the high phosphorus hay can be explained, in part, as a difference in availability of the phosphorus in the two hays due to a difference in the ratio of the amount of phosphorus present to the amounts of substances like iron and aluminum capable of forming insoluble phosphates in the intestinal tract and thus interfering with phosphorus assimilation.

More work needs to be done in extending our knowledge of the chemical quality of feeds by the use of methods other than those now in common practice for feed analysis. This study represents only a preliminary and fragmentary method of attack. The work should be repeated with animals having digestive tracts suited to consuming large amounts of roughage, as has been pointed out by L. A. Maynard ('41) in reference to our previously published articles. Work is at present under way using an herbivorous animal; also further work with rats using different feeding regimes in an attempt to induce all the animals on the ash diets to eat more and thus to make more nearly normal gains in weight. The authors believe, however, that the objectives of the present study are in line with the recent trend in agricultural and nutritional research as expressed by E. C. Auchter ('39) when he says in connection with the problem of human nutrition, "It is a problem of crop production, of food production . . . . But hitherto we have thought of it too largely in terms of quantity . . . . It would be no revolutionary step for us to think in terms of *nutritional* quality as well."

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ALPHA-TOCOPHEROL REQUIREMENT OF THE MOUSE

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Vitamin E prevents sterility in female mice reared upon synthetic diets (Beard, '25–'26; Bryan and Mason, '40). The sterility was shown by Bryan and Mason to be characterized by death and resorption of the fetuses and to be indistinguishable from that observed in rats upon E-deficient diets (Evans and Bishop, '23; Evans and Burr, '27; Urner, '31). Bryan and Mason ('40) observed no symptoms of neuromuscular disturbance near the end of lactation in the young of females upon synthetic low-E diets, such as are frequently seen in young suckling rats under similar conditions (Evans and Burr, '28; Olcott, '38). They found that male mice on the E-deficient diet failed to develop the testicular degeneration typical of male rats that have been deprived of vitamin E (Mattill and Stone, '23; Mason, '26; Evans and Burr, '27).

This is a report upon (1) the α-tocopherol requirement of the female mouse for the prevention of resorption during gestation; (2) the occurrence of muscular dystrophy in the young of females on E-deficient diets; and (3) the failure of male mice to develop testicular degeneration even after the possibility of storage of vitamin E had been reduced to a minimum.

The pathology of these mice was investigated by Dr. A. M. Pappenheimer ('42).

EXPERIMENTAL

The diets, supplements and methods were similar to those used for rats (Goettsch and Pappenheimer, '41).

Diets and supplements. The diets (I and II), similar to those used by Evans and Burr ('27), had the following percentage composition:[1] casein[2] 29.1 (26.6); cornstarch, raw 36.3 (33.3); lard 20.0 (18.3); yeast, bakers' dried 9.0 (16.6); salt mixture (Hawk and Oser, '31) 3.6 (3.4); and cod liver oil[3] 2.0 (1.8). Diet II containing more yeast was used during the period of lactation. The diets were freshly prepared every week.

The stock diet had the following percentage composition: whole wheat 55.5; wheat germ 10.0; casein 10.0; whole milk powder 15.0; $CaCO_3$ 1.5; NaCl 1.0; cottonseed oil[4] 5.0; and cod liver oil 2.0.

Three preparations of α-tocopherol were employed: synthetic dl-α-tocopherol acetate[5] and natural and synthetic α-tocopherols.[6] The tocopherols were dissolved in sesame oil. The solutions were prepared by weight in such proportions that 0.25 mg. of α-tocopherol was contained in 1 drop, and stored at 4°C.; they were used within 1 week of preparation.

METHODS

Young female mice from a stock colony maintained on a commercial dog food[7] were reared on diet I from the time of weaning (21 days). They were weighed every fifth day on a horn pan balance. Vaginal smears were made daily. At about 60 days of age, they were bred with normal males during estrus. If litters were cast, the females were bred until the occurrence of gestational resorptions.

[1] The figures for diet I are those which immediately follow the name of the ingredient; the figures for diet II are given in parentheses.

[2] Merck's technical.

[3] Mead Johnson.

[4] Wesson.

[5] Ephynal, of Hoffman-La Roche, Inc., through the courtesy of Dr. F. Gudernatsch.

[6] Merck and Co., through the courtesy of Dr. J. M. Carlisle.

[7] Purina Chow.

Whenever a gestation resulted in resorption of fetuses, the mice were bred again and given orally, on or before the fifth day after mating, single doses of wheat germ oil or of α-tocopherol. Observations were made upon the number of litters, litter size, and young born alive, which survived the early lactation period and developed muscular dystrophy. After the period of lactation the females were bred to determine the number of litters produced by a single dose of vitamin E.

The young which survived lactation were given diet I. Growth, reproduction and the response to α-tocopherol were observed in the females as in the first generation. Their progeny became the third generation. Seven generations were reared upon diet I with sufficient vitamin E to permit the birth of living young and their survival.

Some males and females of the third generation were reared on the stock diet containing wheat germ and cottonseed oil [8] as sources of vitamin E. Female offspring, reported as the first generation, were reared on diet I and their fertility recorded.

Procedure during gestation. It was more difficult to determine the occurrence of positive mating in the mouse than in the rat (Evans and Burr, '27), for in the mouse the plug apparently does not remain in place so long, nor are sperm often found in smears after the plug has dropped out. By examining the mated mice several times during the day it was possible to observe the plug in about two-thirds of the positive matings; in the other third, pregnancy was determined by the absence of estrus and the finding of the "placental sign" in the daily smears. In all pregnancies, implantation was indicated by the presence of erythrocytes in the smear on the twelfth day after mating. Failure of implantation after positive mating occurred in 32 (7%) of 444 gestations on diet I.

Litters were cast on the twentieth day of pregnancy. In resorption gestations there was a gradual loss of 2 to 8 gm. in body weight from the eighteenth to twenty-second day.

[8] See footnote 4, page 514.

Procedure during lactation. Whenever there were living young, the female was given diet II and permitted to suckle them. Record was made of the number of young which survived the first 10 days of lactation, and during the rest of the period the mice were observed daily for symptoms of muscular dystrophy. Any mice which appeared to be abnormal were killed, autopsied, and specimens preserved for histological examination. After the period of lactation the females were given diet I, and bred again.

At the termination of the experiments, forty females and their young were killed during different stages of lactation and examined.

Males. Males of the second to the seventh generations were reared on diet I. Some were given in addition, 1 or 2 drops of wheat germ oil daily or 1.0 mg. of α-tocopherol per week. They were weighed every fifth day and bred after the sixtieth day with normal females in estrus. Some were under observation during their life span, which rarely exceeded 400 days. At autopsy of males that had been killed, smears from the vas deferens in Locke's solution were examined for motility of sperm, and specimens of testes, muscle, and occasionally central nervous system were preserved.

As soon as it became apparent that the males never developed testicular degeneration under these conditions, males and females were reared together on diet I and not separated until the females were pregnant. The age at which males matured was thus determined.

RESULTS

Growth and estrus in female mice on diet I. Growth, age at the time of first estrus, and the length of estrus cycles of females of all generations reared on diet I were similar to those of controls on the stock diet. The mean body weight of thirty-five females at 60 days of age was 25.0 ± 3.4 gm. The first estrus occurred at 32.6 ± 4.6 days at a body weight of 18.1 ± 2.5 gm. The first estrus cycle varied between 6 and 14 days in length, but thereafter the cycles were regular and

of 4 to 6 days' duration (Allen, '22). In 132 cycles, the mean length was 5.5 ± 1.7 days. These findings are in accord with those observed in female rats on synthetic E-low diets (Evans and Bishop, '23).

Fertility on diet I. As proposed by Bacharach and Allchorne ('38), this was expressed as the per cent of gestations which resulted in the birth of young, regardless of litter size or number of living young.

The fertility of female mice that were reared on diet I from the time of weaning (twenty-first day) is shown in table 1. It will be seen that the fertility varied from 0 to 62%.

TABLE 1

Fertility of female mice on vitamin E-low diet I; showing effect of maternal diet on fertility of offspring.

NUMBER OF GENERATION	NUMBER OF			PER CENT FERTILITY	DIET OF MOTHER DURING LACTATION	SINGLE DOSE OF α-TOCOPHEROL GIVEN MOTHER AT BEGINNING OF GESTATION
	Mice	Gestations	Litters			
						mg.
1	15	13	0	0	Purina Chow	0
	13	13	8	62	Stock diet	0
2	23	22	0	0	Diet II	*
	14	14	2	14	Diet II	0.5
	12	12	5	42	Diet II	1.0
3	44	42	4	10	Diet II	0.5
4	20	20	0	0	Diet II	1.0
5	8	8	0	0	Diet II	1.0
	9	8	0	0	Diet II	2.0
6	6	6	3	50	Diet II	1.0
7	8	7	1	14	Diet II	1.0

* Mother given 25 drops of wheat germ oil.

Since the fertility appeared to be influenced by the amount of vitamin E which was obtained and stored by the young during gestation and lactation (Mason and Bryan, '38), data on the diet of the mother and the amount of α-tocopherol given to her in a single dose at the beginning of gestation were included in the table. When the females with litters were subsequently bred there were only two second litters, both in the group

in which the mothers had been fed the stock diet. From table 1 it is evident that the fertility of sixth and seventh generation mice did not differ appreciably from that of the second.

Curative effect of α-tocopherol. The response of sterile female mice on diet I to single doses of α-tocopherol at the beginning of gestation is shown in table 2. Since there were

TABLE 2

Prevention by α-tocopherol of resorption during gestation in mice of proved sterility on vitamin E-low diet I.

AGE OF MOUSE	α-TOCOPHEROL: SINGLE DOSE AT THE BEGINNING OF GESTATION		NUMBER OF		PER CENT FERTILITY
	Kind	Dose mg.	Gestations	Litters	
3–6 months	*Natural*	0.25	13	8	62
		0.50	10	9	90
3–6 months	*Synthetic*	0.25	6	3	50
		0.50	11	8	73
		1.00	6	5	83
		2.00	4	4	100
3–6 months	*Synthetic acetate*	0.25	12	6	50
		0.50	41	33	80
		1.00	20	18	90
7–12 months	*Synthetic acetate*	1.00	12	6	50
		2.00	12	7	58
		5.00	19	18	95

no apparent differences among them, the data obtained from the seven generations of mice were combined in this and the following tables. It will be seen that, as in the rat (Goettsch and Pappenheimer, '41), the three preparations of α-tocopherol gave essentially the same results: 0.25 mg. induced litters in seventeen (55%) of thirty-one mice; 0.50 mg., in fifty (80%) of sixty-two mice; at 1.0 to 2.0 mg., the incidence was higher. As in the rat, the biological variation was very great. In older mice the requirement for vitamin E was greater, as has been reported for the rat (Emerson and Evans, '39).

The minimum amount of α-tocopherol which permitted the birth of young to at least 85% of the mice was approximately 0.5 to 1.0 mg. under the given experimental conditions. This

value is higher than that reported for the mouse by Bryan and Mason ('40) and is roughly one-third of the amount, 1 to 3 mg., required by the rat for the birth of living young (Evans, Emerson and Emerson, '36; Karrer and Keller, '38; Bacharach, '38; Goettsch and Pappenheimer, '41).

Calculated by the surface area formula of Meeh and the constants of Rubner (Lusk, '28), the body surface of a 200-gm. rat is 311.2 cm². ($9.1 \times 200^{2/3}$), and that of a 25-gm. mouse, 97.5 cm². ($11.4 \times 25^{2/3}$). The body surface of the mouse is therefore about one-third as great as that of the rat. These data suggest that per body surface area, the α-tocopherol requirement for the prevention of resorption during gestation is the same in the rat and mouse.

Second litters were rarely born after single doses of α-tocopherol, and there were no third litters.

Litter size. The litters varied in size from one to fourteen mice, averaging 6.7 gm. for the controls on the stock diet and for mice on diet I that were fed 1.0 mg. α-tocopherol. It will be seen in table 3 that litters tended to be smaller both

TABLE 3

Influence of α-tocopherol upon litter size, the number of young born alive, surviving the early lactation period, and developing muscular dystrophy.

	MG. OF α-TOCOPHEROL						
	Single dose at beginning of gestation						STOCK DIET E
	0 [1]	0.25	0.50	1.0	2.0	5.0 [2]	
No. of litters	25	17	50	29	10	18	10
No. of young	125	104	309	195	56	95	67
Av. young per litter	5.0	6.1	6.2	6.7	5.6	5.3	6.7
No. living young	110	93	272	180	45	88	65
Per cent	88	89	88	92	80	93	97
No. surviving 10 days	64	68	207	126	40	50	63
Per cent	58	73	76	70	89	57	97
No. with muscular dystrophy	7	8	27	20	4	8	0
Per cent of those surviving	11	12	13	16	10	16	0

[1] First litter fertility.

[2] Older animals.

in older mice and in those that received lesser amounts of vitamin E. The average birth weight was 1.34 gm. for 181 mice of the fourth and fifth generations.

Number of living young. It will be seen in table 3 that in all litters on diet I, 80% or more of the young were living at the time that the litters were found, regardless of the amount of α-tocopherol.

The cause of death in the newborn mice was undetermined but lesions of muscular dystrophy were found in thirteen of twenty-two that were examined histologically.

Survival of young during lactation. As shown in table 3, 70 to 89% of young mice born to females on diet I with 0.25 to 2.0 mg. of α-tocopherol survived the first 10 days of lactation. The young of older females and those born as a result of "first litter fertility" did not survive so well.

Of sixty-three mice that did not survive and were examined histologically, ten presented typical lesions of muscular dystrophy.

Muscular dystrophy. Although the young mice were observed daily during lactation for symptoms of "paralysis" such as occur under similar circumstances in the rat (Evans and Burr, '28), these never appeared (Bryan and Mason, '40). A few mice died, some developed queer darting movements, and some became emaciated. At autopsy, the muscle seldom appeared abnormal, but occasionally it had a cheesy consistence or was streaked with yellowish grey. Microscopically there was hyaline necrosis of the muscle fibers with early calcification and active regeneration. Muscle lesions were occasionally found in older mice. There were no other changes of significance in either the brain, cord, peripheral nerves, bones, nor in any of the viscera examined (Pappenheimer, '42).

Since the mice presented no symptoms it was impossible to tell how many of the young of the second to the seventh generations had the disease during lactation and spontaneously recovered. The incidence of muscle disorder, given in table 3, might have been greater if all of the young had been killed and

examined. The disease occurred sporadically in about 15% of the young, rarely affecting all members of a litter. In the rat under similar conditions muscular dystrophy occurs in 90% of the young from the seventeenth to twenty-third day of lactation, with a mortality of 35%, and is prevented by the administration of 0.5 mg. of α-tocopherol on the fifteenth day (Goettsch and Pappenheimer, '41). The preventive effect of α-tocopherol in mice was not investigated.

Growth of male mice on diet I. Growth of males of all generations reared on diet I was similar to that of controls on the stock diet. The mean body weight at 60 days of age of fifteen males on diet I was 29.0 ± 2.9 gm. In twelve littermates that were given wheat germ oil or α-tocopherol the mean body weight at 60 days was 28.7 ± 3.0 gm. Subsequently there was no difference in the rates of growth between the various groups.

With respect to growth, male mice respond differently from male rats to synthetic E-low diets, for in the latter, growth is invariably retarded and is restored by vitamin E (Evans and Burr, '27).

TABLE 4

Fertility of male mice on vitamin E-low diet I.

	GENERATION					
	2	3	4	5	6	7
Number of mice	21	21	14	5	3	3
Number siring litters	21	21	14	5	3	3
First fertile mating						
Number of mice	6	8		5	3	3
Age in days, av.	60	66		62	58	45
Age in days, extremes	(40–81)	(55–81)		(55–75)	(36–76)	(39–59)
Last fertile mating						
Number of mice	13	11	7			
Age in days, av.	190	216	182			
Age in days, extremes	(128–299)	(144–376)	(105–255)			
Age at time of death						
Number of mice	13	11	7			
Days, av.	298	283	283			
Days, extremes	(154–341)	(166–486)	(169–392)			

Fertility of males on diet I. As will be seen in table 4, all of the males of the second to the seventh generations were fertile. These results, though in complete accord with those of Bryan and Mason ('40) are surprising inasmuch as male rats of the second and third generations are invariably sterile from the beginning of sexual maturity (Evans and Burr, '27).

Sexual maturity, as judged by the ability to sire litters, occurred at an average age of 60 days. The length of the fertile period varied greatly but did not appear to be shorter in the males on diet I than in those given vitamin E. Towards the end of the breeding period, males occasionally appeared to be sterile, as indicated by the finding of plug without implantation. However, at the time of death, both motile sperm in the vas deferens and histologically normal testes were invariably found.

SUMMARY

1. Seven generations of mice were reared on a synthetic E-low diet with just sufficient α-tocopherol fed to the females to permit the birth of living young and their survival.

2. In such mice there is neither retardation in growth nor any change in the age when the first estrus appears and in the length of the estrus cycle. Females are sterile when α-tocopherol is withheld.

3. Under the conditions described, the administration of 0.5 to 1.0 mg. of α-tocopherol at the beginning of gestation results in the birth of young to at least 85% of the females.

4. Lesions without symptoms of muscular dystrophy may occur at any age in the mouse; occasionally in the newborn and in adults, and to an extent of 15% in the young during the first 35 days of life.

5. Males retain their fertility and never develop testicular degeneration.

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CERTAIN RELATIONSHIPS OF AVITAMINOSIS A TO VITAMIN C IN THE YOUNG BOVINE[1]

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The decrease in ascorbic acid content of tissues in the vitamin A deficient dairy calf has been shown by Phillips et al. ('38) and King ('40). Sure, Theis and Harrelson ('39) have demonstrated a decreased ascorbic acid content of the tissues and blood plasma of the vitamin A deficient rat. The investigations reported here were undertaken to study the relationship between vitamin C and the characteristic effects of vitamin A deficiency as described by Walker ('36), King ('40), Moore et al. ('39, '40) and Phillips and co-workers ('38). It was also desired to study the relationship of the blood levels of vitamins A and C, and the effects of avitaminosis A on urinary vitamin C excretion.

EXPERIMENTAL PART

The data reported were taken from observations made on eleven calves, eight Holsteins and three Guernseys, which were maintained on the basal vitamin A deficient ration (Walker, '36) for 5 to 8 months. They were fed either the basal ration alone, or the basal ration supplemented by either crystalline carotene in cottonseed oil, or vitamin A as shark liver oil. Carotene and vitamin A were fed in amounts equivalent to 0–100 μg. and 0–18 μg., respectively, per kilogram of body

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weight per day. The levels fed were such as to completely prevent or produce varying degrees of vitamin A deficiency.

Weekly analyses of blood plasma for vitamins A and C, and carotene, were made by methods previously used in this laboratory (Phillips, Lundquist and Boyer, '41). Ophthalmoscopic observations for papillary edema, measurements of intracranial pressure, and determinations of the vitamin C content of the cerebrospinal fluid were made at frequent intervals on several of the calves. Intracranial pressure measurements were made by use of a water manometer, a puncture being made at the occipito-atlanto joint following the technique developed by Moore and Sykes ('41).

When increased pressure and papilledema developed, vitamin C was given to several of the calves to see if any remission of these conditions could be obtained.

In these experiments the blood plasma vitamin A values of the calves not receiving vitamin A supplements decreased to a low level of 0.03–0.05 μg. per cubic centimeter after 2 weeks on experiment. It was interesting to note that definite papilledema did not develop until after an average of 5 weeks on experiment, and a severe deficiency associated with increased intracranial pressure did not occur until an average of 8 weeks after low levels of blood vitamin A were observed. Thus the drop in blood plasma vitamin A content precedes by several weeks the development of these pathological conditions. Supplementation with subminimal amounts of vitamin A or carotene delayed the onset of the deficiency, but again low levels of plasma vitamin A were reached several weeks before marked deficiency symptoms developed. Grouping of the vitamin C analyses according to the vitamin A content of the blood plasma (table 1) showed that a definite relationship existed between the amounts of vitamins A and C present. The differences seem to be somewhat more pronounced with the Guernsey than with the Holstein breed.

Measurements of the ascorbic acid content of the cerebrospinal fluid showed that it normally contained from five to ten times as much ascorbic acid as the blood plasma. A striking

relation was found to exist between the ascorbic acid content of the cerebrospinal fluid and the degree of increased intracranial pressure (table 2). The drop in the vitamin C content of the cerebrospinal fluid with the increased intracranial pressure was much more pronounced than the corresponding decrease in the plasma ascorbic acid.

TABLE 1

Relation between blood levels of vitamins A and C.

PLASMA VITAMIN A	PLASMA VITAMIN C	
	Holstein calves	Guernsey calves
μg./cc.	*mg.%*	*mg.%*
0.02–0.05	0.207 (64)[1]	0.202 (17)[1]
0.06–0.10	0.254 (54)	0.254 (36)
0.11–0.18	0.272 (23)	0.304 (12)

[1] Figures in parentheses indicate number of analyses.

TABLE 2

Relation of intracranial pressure to vitamin C content of the cerebrospinal fluid and blood plasma.

INTRACRANIAL PRESSURE	AVERAGE PAPILLARY EDEMA	VITAMIN C CONTENT			
		Cerebrospinal fluid		Blood plasma	
		Average	Range	Average	Range
mm. H_2O	*diopters*	*mg.%*	*mg.%*	*mg.%*	*mg.%*
100–200 (normal)	0	2.30	1.64–2.42 (9)[1]	0.24	0.15–0.36 (15)[1]
200–300 (increased)	2.0	1.43	0.64–1.94 (21)	0.22	0.12–0.35 (24)
Over 300 (markedly increased)	2.6	1.28	0.64–1.84 (8)	0.18	0.10–0.29 (12)

[1] Figures in parentheses indicate number of analyses.

Several experiments were made in an attempt to ascertain if the administration of vitamin C would bring about a lowering of the intracranial pressure. Four calves were given from 5 to 6 gm. of vitamin C over a period of a week after they had developed an average elevated pressure of 310 mm. of water. In three cases the vitamin C administration was followed by a drop in pressure to an average of 180 mm. of water. In the

fourth case the pressure was not significantly lowered. Administration of ascorbic acid for periods longer than 1 week was impractical because of the moribund condition of the calves. The vitamin C content of the cerebrospinal fluid increased from 0.64 and 0.99 mg. per cent to 1.50 and 1.66 mg. per cent, respectively, in two calves in which pressure decreased during ascorbic acid administration. The calf which showed no diminution of cerebrospinal pressure had an increase in the vitamin C content of the cerebrospinal fluid from 1.63 to 2.01 mg. per cent during the period of ascorbic acid administration.

A further experiment was conducted by allowing one of the calves which had responded to vitamin C administration to again develop an increased intracranial pressure. When the pressure had reached 480 mm. of H_2O, 16 gm. of vitamin C was given over a 12-day period. No decrease in pressure occurred, although the vitamin C content of the cerebrospinal fluid was brought back to a normal value of 2.40 mg. per cent. The calf was in an extremely deficient state. Subsequent administration of large amounts of shark liver oil reduced the pressure to 300 mm. after 10 days and 170 mm. after 20 days and maintained the vitamin C content of the cerebrospinal fluid.

The decreased vitamin C in the blood and tissues found in the vitamin A deficient animal may have been due to either decreased synthesis or increased excretion of the vitamin. To ascertain if there was a decreased excretion of ascorbic acid in a vitamin A deficiency, which would indicate a decreased rate of synthesis, twenty-four rats of 30 days of age were placed on a diet low in vitamin A. The rats were fed the basal diet having a percentage composition of dextrin 68, alcohol extracted casein 18, brewer's yeast 7.9, irradiated yeast 0.1, salts 4, and corn oil 2. Twelve rats received the diet unsupplemented and twelve received in addition 1 drop of shark liver oil containing 15,000 I.U. per gram twice weekly. All rats received 2–3 mg. of α-tocopherol twice weekly. After the growth of the vitamin A deficient rats had reached a

plateau or begun to decrease, the animals were placed in metabolism cages, and urinary vitamin C determined on the daily excretions collected over a 2-week period. The results showed a marked decrease in vitamin C excretion by the vitamin A deficient rats. Deficient male rats excreted an average of 0.19 mg. of vitamin C per kilogram of body weight as compared to 0.49 mg. per kilogram for control males. Deficient females excreted 0.16 and control females 0.52 mg. per kilogram of body weight, respectively.

DISCUSSION

The striking relationship found to exist between the decreased vitamin C content of the cerebrospinal fluid and the increased intracranial pressure suggests that the low vitamin C is in some way associated with the increased pressure. The lowering of pressure in three calves following vitamin C administration lends support to this suggestion, but the failure to secure this in the fourth calf, and in the calf in which increased pressure was permitted to recur makes it impossible to assign a specific role to vitamin C in this respect. King ('40) has also suggested that vitamin C may be involved in the pathological changes characteristic of vitamin A deficiency. In relation to the cause of the increased intracranial pressure, Moore and Sykes ('41) found no abnormality in various blood and urine analyses, and no pathological changes of the choroidal plexus, arachnoid villi, and colloidal blood plasma osmotic pressure. The decreased vitamin C of the cerebrospinal fluid might be related to an increase in the volume of cranial contents postulated as a possible cause of the increased pressure (Moore and Sykes, '41); the increased volume of cranial contents being due to an actual volume increase, or to a relative overgrowth of the nervous system as indicated by Wolbach and Bessey ('40). The results of these experiments suggest that further studies should be made on the effects of ascorbic acid on the bone anomalies and other tissue changes characteristic of avitaminosis A that occur in rats (Wolbach and Bessey, '40), dogs (Mellanby, '41) and calves.

It is of interest to note that the development of avitaminosis A symptomatology and pathology does not occur until the plasma vitamin A has dropped to the low level of 0.05–0.07 μg. per cubic centimeter, or less, and remained there for several weeks. Although development of vitamin A pathology is related to the blood level of the vitamin, chronic low blood plasma levels may exist for some time before marked pathology develops.

The pronounced decrease in urinary excretion of vitamin C by the vitamin A deficient rat indicates that the decrease in the blood and tissue vitamin C of the rat, and in all probability of the cow, results not from increased excretion, due to possible kidney damage, but from decreased synthesis of this vitamin within the animal body. This decreased synthesis of vitamin C in vitamin A deficiency is of particular interest in view of the results obtained by the treatment of bovine sterility with ascorbic acid (Phillips et al., '41).

SUMMARY

Various degrees of vitamin A deficiency have been produced in the dairy calf, and the relationship of the deficiency to vitamin C studied. Urinary excretion of vitamin C by the vitamin A deficient rat has also been determined. These studies have shown:

1. The level of blood plasma ascorbic acid in the dairy calf is contingent upon the level of vitamin A, particularly so when the vitamin A values fall below 0.10 μg. per cubic centimeter.

2. The development of vitamin A pathology occurs only after the blood plasma levels of vitamin A have decreased to a low level of 0.05 to 0.07 μg. per cubic centimeter or less and remained there for several weeks.

3. The increased intracranial pressure observed in the calf suffering from vitamin A deficiency is paralleled by a marked decrease in the ascorbic acid content of the cerebrospinal fluid. The vitamin C content of the cerebrospinal fluid of the cow is normally five to ten times that of the blood plasma.

The administration of vitamin C to A-deficient calves was accompanied by a rise in the vitamin C content of the cerebrospinal fluid, a reduction of the cerebrospinal pressure occurring in three out of five cases. It appears that vitamin C is in some way associated with the increased intracranial pressure.

4. The urinary excretion of vitamin C in the vitamin A deficient rat is greatly reduced, thus indicating that the lowered blood and tissue vitamin C is the result of impaired synthesis.

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